Functional Validation of MicroRNAs Correlated with Second Biochemical Recurrence After Post-Prostatectomy Radiation in Prostate Cancer

Undergraduate Research Thesis

Presented in partial fulfillment of the requirements for graduation *with honors research distinction* in Neuroscience in the undergraduate colleges of The Ohio State University

by

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Abstract

Prostate cancer (PCa) is one of the most common cancers and the second leading cause of cancer-related death in the U.S. (American Cancer Society, 2017). While treatment strategies for PCa rely primarily on radical prostatectomy (RP), more than one-third of men receiving this treatment experience relapse of the cancer phenotype. Also known as biochemical recurrence, this relapse is defined by a PSA rise of >.2 ng/mL at least twice, or clinical progression demonstrated by local, regional, or distant recurrence as noted via imaging (CT, PET, or bone scan) (Bell, 2015). Patients experiencing this biochemical failure therefore require salvage radiation therapy (RT), a form of treatment given after disease recurrence (Uchio, 2010). However, a small subset of these patients experience a second biochemical recurrence after receiving salvage RT. These patients therefore represent a high-risk cohort with aggressive biological disease, which cannot be predicted with current clinical standards. This creates a lack of optimized treatment strategies to prevent under-treatment in patients with aggressive disease and over-treatment in patients who will not benefit from additional therapy but suffer toxic side effects. Our group previously identified nine microRNAs (miRNAs) that could predict biochemical recurrence after salvage RT in a cohort of PCa patients (n=43) (Bell, 2015). As predictive biomarkers, these miRNAs could provide a method of identifying and classifying patients based on response to RT. Of these, *miR-628-3p, miR-320e, miR-508-3p, miR-598, miR-601,* and *miR-563* have been shown to play a role in PCa while *miR-1193, miR-4516,* and *miR-626* are novel in PCa but linked to roles in other cancers (Srivastava, 2014; Hsieh 2013; Peng, 2011; Corcoran, 2014; Hessvik, 2012; Haj-Ahmad, 2014; Chowdhari, 2014; Riaz, 2013; Li, 2016). To our knowledge, there are not yet any publications indicating a role for any of these nine miRNAs in radiation resistance. The purpose of this study is to identify pathways of radiation resistance in PCa and characterize miRNAs previously linked to biochemical failure after salvage RT. We hypothesized that miRNAs previously identified to be predictive of salvage RT response may play a role in radiation resistance or cancer progression. Seven previously identified miRNAs were

screened *in vitro* to determine their possible mechanism(s) in radiation resistance. Of these seven miRNAs implicated in PCa radiation resistance, *miR-508-3p* and *miR-1193* were consistently correlated with tumor inhibitory effects; both *miR-508-3p* and *miR-1193* decreased cell proliferation, cell viability, and inhibited colony formation in response to radiation. These miRNAs therefore represent potential therapeutic targets that provide insight to the mechanisms of cancer progression in PCa patients.

Significance

Our previous work was the first to show a correlation between miRNAs and second recurrence in PCa patients receiving salvage radiation. This work represents the first functional characterization of these miRNAs in order to identify relevant pathways of radiation resistance in PCa patients. The results of this study provide a potential method of classification of patients predicting response to treatment, thereby identifying which patients should be treated with salvage RT as well as those that may benefit from earlier, more aggressive treatment. The identification of novel biomarkers of biochemical recurrence will potentially offer insight into the functional relevance of these miRNAs implicated in PCa radiation resistance as effective therapeutic targets, as well as a more thorough comprehension of mechanisms of cancer progression and radiation resistance in PCa. This project is innovative because all selected miRNAs would represent novel biomarkers in PCa. Further, mechanisms of radiation resistance in PCa are not well-understood, but are more thoroughly explored in this study through the functional validation of miRNAs implicated in PCa radiation resistance.

Optimized treatment strategies can potentially be developed by targeting miRNAs directly or indirectly through their targeted genes. Targeting miRNAs directly, including through expression vectors encoding the miRNA sequence, small molecule inhibitors, and antisense oligonucleotides, has been studied extensively *in vitro* and *in vivo,* yet requires additional development before being implemented clinically (Li, 2014). Other important follow-up studies would be required beyond the identification of miRNA targets and development of targeted delivery methods, including analyzing appropriate dosing of miRNA therapies and *in vivo* studies.

Introduction

Prostate Cancer

Prostate cancer (PCa) is a deadly disease affecting 1 in 7 American men – the second most lethal cancer in men overall after skin cancer (American Cancer Society, 2017). There are more than 200,000 new cases of PCa and more than 27,000 deaths due to PCa each year (American Cancer Society, 2017). PCa is typically detected using the prostate specific antigen (PSA) blood test, allowing early, non-invasive detection, followed by biopsy to prove the presence of cancer. Tissues are then staged by Gleason score, a histological grade assigned based on the similarity of appearance of cancerous tissue to normal tissue. Primary treatment may include interstitial brachytherapy, external beam radiation, androgen deprivation therapy, and/or radical prostatectomy (RP) (Kollmeier and Zelefsky, 2012).

Limitations of Current PCa Treatment Strategies

The multitude of therapeutic options combined with the lack of available data on therapeutic efficacy for different patient subgroups creates difficult treatment decisions for physicians and patients. While the PSA test often allows early detection, it lacks discriminative capability. Early detection may lead to the treatment of clinically insignificant disease which could have been managed with active surveillance. For example, Parker et. Al (2006) determined that less than 2% of PCa patients with a Gleason score below 6 actually died of PCa. Together, the current clinical standards of PSA and Gleason score lack the ability to accurately provide risk stratification of PCa patients (McGrath, 2016). This confusion leads to the potential for under-treatment of aggressive disease and therefore higher mortality rates, but also for overtreatment of non-threatening disease. Importantly, PCa treatment causes long-term toxic side effects and a reduction in quality of life, which could be avoided in indolent disease. Current clinical standards lack a reliable screening method to discriminate between patients who will experience indolent disease from those who face life-threatening, aggressive disease, leading to a dire need for better methods of classifying patients.

PCa varies from indolent subtypes to far more aggressive disease phenotypes. Among those patients who undergo RP, one-third fails treatment and requires salvage radiation therapy (Uchio, 2010). In addition, previous studies have shown that greater than 40% of this subset of patients fail salvage radiation therapy (RT), experiencing a second biochemical recurrence (Uchio, 2010). Further, given the variety of disease states and treatment options in PCa, there is currently no reliable method of predicting patients' response to treatment, such as via molecular biomarkers. With that said, there is a vital need for prognostic biomarkers to predict patients' overall outcome as well as predictive biomarkers to predict patients' response to specific treatment. This could provide a method to accurately stratify patients by disease aggressiveness and response to treatment to improve quality of life and mortality outcomes for PCa patients.

Clinical Impact

Despite the lack of predictive and prognostic biomarkers currently available, biomarkers represent an opportunity to improve diagnosis, prediction of patient outcome, response to therapy, and risk stratification. In addition, there are currently no miRNA biomarkers utilized in PCa, yet they offer many benefits. MiRNAs are useful due to their ability to be detected in biofluids, providing the opportunity for non-invasive clinical testing. MiRNAs are stable in formalin-fixed, paraffin-embedded (FFPE) tissue due to their small size, allowing accurate profiling of human clinical biopsied samples (Smith, 2017). Further, miRNA biomarker tests are cost-effective, providing realistic clinical application (Kapoor, 2016). MiRNAs therefore represent potential for precise and personalized medicine as diagnostic, predictive, and prognostic biomarkers in PCa.

Radiotherapy and Radiation Resistance

Ionizing RT is used to treat cancer due to its ability to cause DNA damage, preventing cancer cells from growing and dividing. Although RT affects both healthy and cancerous cells, it can be targeted to a localized area, as compared to the more global approach of chemotherapy. Further, because cancer

cells divide more quickly and self-repair less than non-cancerous cells, they are more vulnerable to RT than healthy cells (Baskar, 2014). RT can work by either directly causing DNA damage and killing cells, or indirectly by inducing free radical formation which eventually kills cells. The effects of RT are delayed and complex, working through a variety of mechanisms. Specifically, RT intends to produce cell death through the infliction of DNA damage; however, this DNA damage can take the form of either single- or double-stranded breaks. The double-stranded break (DSB) is more deleterious, yet occurs less frequently than the single-stranded break (SSB). Further, DNA damage in cancer cells is more difficult to repair due to the dense clustering of damage (Lomax, 2013). Unrepaired DSBs lead to cell death, the majority of which occurs via apoptosis or mitotic catastrophe. Less frequently, tumor growth may halt due to the mechanisms of necrosis, senescence, or autophagy (Baskar et al., 2012). Each of these mechanisms involves distinct pathways; for example, the apoptotic pathway primarily works through the activation of caspase enzymes, which in turn activate endonucleases which degrade chromosomal DNA and lead to the formation of apoptotic bodies (Elmore, 2007).

However, despite the potential efficacy of RT, many patients do not respond, as exemplified in our cohort. Although less efficacious, cancer cells still maintain DNA damage repair pathways which contribute to the resistance of cancer cells to RT (Lomax, 2013). The main pathway by which DSBs are repaired is through non-homologous end joining (NHEJ), which rejoins the ends of the separated DNA strands. Briefly, upon recognition of the DNA ends, an NHEJ complex assembles, including the Ku heterodimer, DNA-dependent protein kinases, and other essential factors. This complex bridges the ends of the strands, which are then processed by enzymes which make the ends of the DNA strands able to be re-joined. Finally, these ends are ligated by DNA Ligase IV, leading to the reparation of the break (Davis, 2013). Additionally, DSBs can be repaired through homologous recombination, in which the ends of a DSB are resected and processed to generate 3’OH single strands. These ends associate with the

Rad51 enzyme, which identifies and invades an identical sequence (usually on a sister chromatid) to promote DNA synthesis via DNA Polymerase (Sonoda, 2006).

In addition to inherent mechanisms of DNA damage repair, other factors contribute to the relative resistance of cancer cells to RT. While mechanisms of radiation resistance are not fully understood, many theories center on the relative stemness of cancer cells, providing their ability to self-renew and produce heterogeneity of tumor cell subtypes (Pajonk et al., 2010). RT intends therefore to eradicate these cancer stem cells (CSCs) through irreparable double-stranded DNA breaks. However, the efficacy of RT depends on a number of factors, including the relative quantity of CSCs present in a tumor, overall tumor volume, rate of tumor cell repopulation, tumor microenvironment such as hypoxia, and ability to repair DNA damage. Intrinsic ability to respond to DNA damage directly involves a variety of DNA damage repair pathways and their epigenetic modulation (Willers, 2013). Additionally, because damage induced by irradiation often requires several cell divisions to cause cell death, other cell survival, cell cycle checkpoint, and cell death pathways are also involved in radiation response. Epigenetic modulators of these genes and pathways such as miRNAs therefore have an inherent role in response to RT (Metheetrairut, 2013).

MicroRNAs

Biogenesis

MicroRNAs (miRNAs) are small, single-stranded non-coding RNA molecules which downregulate gene expression and therefore downstream protein production. Found in the genomes of a variety of plants and animals including humans, miRNAs have been estimated to regulate 60% of genes in the human genome (Friedman, 2009). Biogenesis begins in the nucleus where RNA Polymerases II or III transcribe miRNA genes into primary miRNAs, or pri-miRNAs (Figure 1). These pri-miRNAs are then processed into 70-nucleotide stem loop precursor miRNAs (pre-miRNAs) by the Drosha RNase III endonuclease and double-stranded RNA-binding protein DiGeorge syndrome critical region 8 (DGCR8)

which directs Drosha to a specific cleavage site (Catalanotto, 2016). These pre-miRNAs are then transported into the cytoplasm by transport factor Exportin-5 and RanGTP. Further processing occurs within the cytoplasm, where RNAse III enzyme Dicer cleaves pre-miRNAs into duplexes, or double-stranded miRNAs (ds-miRNA). These duplexes consist of both a passenger strand and a mature or guide strand, which are bound through imperfect base pairing (MacFarlane and Murphy, 2010). An RNA inducing silencing complex (RISC), then loads this ds-miRNA onto Argonaute, which unwinds the two strands of the miRNA duplex, resulting in the incorporation of the single-stranded mature miRNA molecule. The mature miRNA will then be involved in downstream regulation of target mRNA (Catalanotto, 2016).

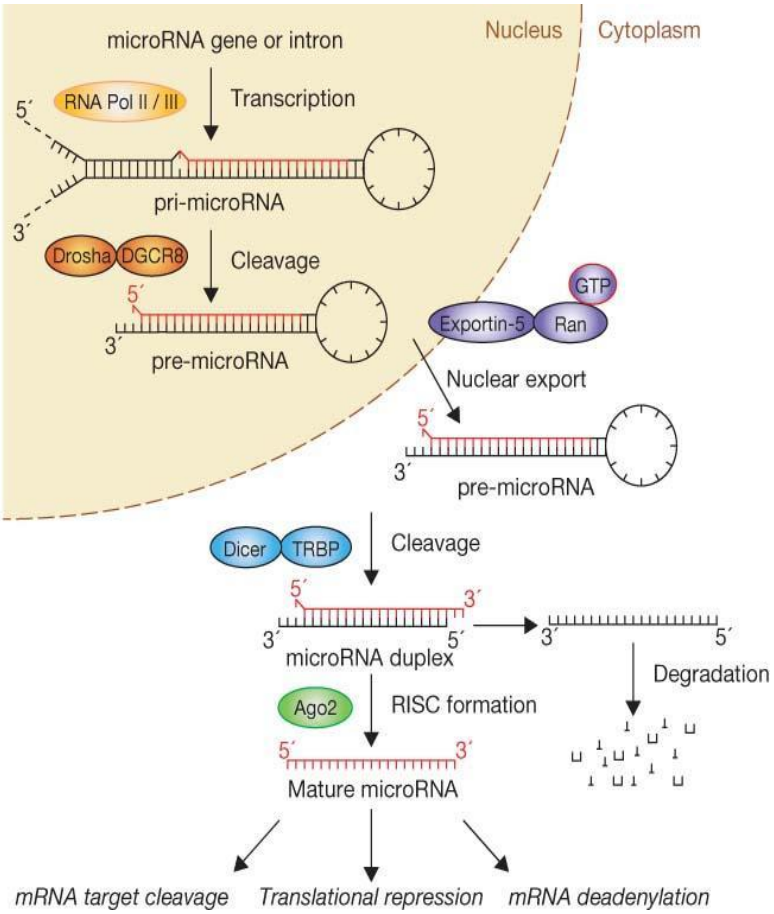

Figure 1. MiRNA biogenesis begins in the nucleus and is transported into the cytoplasm for maturation. Image adapted from Winter, 2009.

Mechanisms

The ultimate function of these mature miRNAs is to regulate gene and protein expression, mainly through inhibition. Once the mature miRNA is loaded into the RISC protein complex, it acts as a guide to identify mRNA targets, to which the guide strand base pairs via its 5' end to the 3' untranslated region (UTR) of its target mRNA. Due to the imprecise complementarity of the guide and target, a single miRNA can target multiple mRNA molecules; likewise, a single mRNA may have multiple miRNA regulators. This regulation occurs through several mechanisms, mainly cleavage by the Ago2 protein, causing mRNA degradation, or through inhibition of translation through various mechanisms. The specific mechanism of silencing appears to depend on the degree of complementarity between the seed region of the guide miRNA and the 3' UTR of its target mRNA, with the miRNA ultimately down-regulating gene expression (MacFarlane and Murphy, 2010).

Implications in Cancer

The diversity of mRNA targets allows miRNAs to influence a variety of biological processes, including cell cycle regulation, cell growth, apoptosis, cell differentiation, and DNA damage response, among other pathways (Chen, 2013). These same pathways are deregulated in cancer, causing normal cells to proliferate uncontrollably and develop the disease phenotype of cancer (MacFarlane and Murphy, 2010). Therefore, miRNAs represent a potential epigenetic mechanism responsible for cancer initiation and progression. MiRNAs were first reported to play a role in cancer in 2002 with the discovery of the deregulation of *miR-15* and *miR-16* in leukemia; the deletion of these miRNAs allowed higher expression of an anti-apoptotic target (Calin et al., 2002). Since then, miRNAs have been identified in many types of cancers, including prostate (Gourdanpour, 2012). The deregulation of these biological regulators provides an opportunity for the identification of novel biomarkers and therapeutic targets in a variety of cancers.

Current Prospects of Biomarkers in PCa

Despite the need for reliable diagnostic, prognostic, and predictive assays in PCa, preliminary research has suggested that molecular biomarkers have great potential for clinical utility. As previously stated, the PSA test lacks specificity and a definite cutoff value for diagnosis. Further, PSA kinetics (including PSA velocity and PSA doubling time) have not been shown conclusively to improve the predictive value or specificity of PSA alone (Sharma, 2016). However, several biomarkers are currently used in clinical practice to assist in the diagnosis of PCa, including *PCA3*, a noncoding RNA found only in prostate tissue which is overexpressed in 95% of PCa cases (AUC .658). Further, ConfirmMDx® (MDxHealth, Inc., Irvine, CA) measures the degree of methylation of *GSTP1, APC*, and *RASSF1*, where negative results of the assay can confirm negative diagnostic value of PCa up to 90% and prevent a second biopsy (Sharma, 2016).

While these biomarkers suggest progress in providing precise diagnostic tools, biomarkers are far more lacking in providing prognostic or predictive value. Currently, treatment decisions cannot be based on PSA kinetics, including PSAV, PSADT, or PSAD. In addition, the *TMPRSS2:ERG* gene fusion does not have prognostic value alone, but may have predictive value of overall survival in metastatic patients undergoing palliative transurethral resection of the prostate, when used in combination with other gene expression markers (Sharma, 2016). While homozygous deletion of the *PTEN* gene has been associated with worse disease stage, suggesting its use as an independent prognostic biomarker, clinical trials have achieved poor responses (Wise, 2017).

However, four gene expression panels are currently available and clinically utilized, including Decipher®, Promark®, Prolaris®, and Oncotype DX®. Altogether, these panels assess 85 different genes. For example, the Decipher® Prostate Cancer Test (GenomeDx Biosciences, San Diego, CA) uses the expression of 22 RNA markers to predict metastasis and mortality in PCa. This tissue-based genomic assessment tests for RNA features of both protein-coding genes as well as small noncoding RNAs, and is designed to help predict the need for salvage or adjuvant RT post-RP (Dalela, 2016). The Promark® test

(Metamark Genetics Inc., Waltham, MA) is an eight-biomarker proteomic assay testing for expression of *CUL2, DERL1, FUS, HSPA9, PDSS2, pS6, SMAD4*, and *YBX1*; expression in tissue is intended to differentiate among indolent versus aggressive PCa. Prolaris® (Salt Lake City, UT, USA) measures cell cycle progression genes and is designed to differentiate among those patients who need active surveillance and those who need more therapy. Finally, Oncotype DX® (Redwood City, CA, USA) is a 12 gene test with a similar function as the Prolaris test. Assessment of the long term potential of these tests to guide treatment decisions is still ongoing (Zhuang, 2016).

Biomarkers capable of predicting response to treatment in PCa are most lacking. However, some progress has been made. As previously mentioned, the Decipher® test predicts the likelihood of metastasis after RP, and Zhao et al. have developed and validated a 24 gene panel which predicts incidence of metastasis in patients receiving RT compared to patients who do not (2016). However, further research needs to be done regarding the clinical implementation of such a panel. Further, there are no currently utilized miRNA biomarkers, suggesting the need for research to develop clinically applicable predictive biomarkers (Zhao, 2016).

Preliminary Data

As previously described, current treatment strategies for PCa are ineffective for a significant proportion of patients (Botticella, 2014). Our study found that after both RP and salvage RT, 44% of men still experienced a biochemical failure (Bell, 2015). The identification of miRNAs linked to biochemical recurrence would provide insight to the pathways and mechanisms leading to radiation resistant PCa. Ultimately, this knowledge could lead to the development of more optimized treatment strategies for PCa patients, especially those with aggressive biological disease. In our previous work, we were the first to report miRNA biomarkers correlated with clinical outcomes in PCa patients treated with salvage RT (Bell, 2015). Therefore, the present study represents the first functional characterization of miRNAs linked to second biochemical recurrence and may offer therapeutic and predictive potential.

Specifically, the Chakravarti lab has shown that 9 miRNAs correlate with biochemical recurrence after RP and salvage RT in PCa patients, including *miR-628-3p, miR-1193, miR-601, miR-4516, miR-320e, miR508-3p, miR-598, miR-626,* and *miR-563* (Table 1) (Bell, 2015). Of these, *miR-1193, miR-4516,* and *miR-626* are novel in PCa (Chowdhari 2014; Riaz, 2013). In particular, *miR-601* and *miR-4516*, when used together with pathological features such as Gleason score and lymph node involvement, may identify patients with high risk of recurrence after salvage RT and may have a great impact on a patient's treatment choice. *MiR-601* is known to downregulate the expression of NF-kβ (Ohdaira, 2009) and *miR-4516* is known to downregulate *STAT3* (Chowdhari, 2014), indicating a potential role in the mechanism of resistance to radiation therapy. Given the already ongoing research on *miR-4516* and *miR-601*, these miRNAs were excluded from further analysis in the present study. However, although the correlation of the remaining seven miRNAs with second biochemical recurrence in patients is now known, possible mechanisms leading to recurrence remain elusive and further testing needs to be performed. Being linked to biochemical recurrence after salvage RT, these seven miRNAs offer an opportunity to better understand pathways of radiation resistance and are the primary focus of the current study.

Hypothesis

We hypothesize that those miRNAs correlated with second biochemical recurrence after RP and salvage RT are playing a role in radiation resistance or sensitivity. The approach to prioritizing the seven available miRNAs was based on their respective hazard ratios, where the hazard ratio confers the probability of an event occurring in the treatment group as opposed to the control group; in the present context, a high hazard ratio is indicative of worse clinical outcome. Specifically, those miRNAs with high hazard ratios correlated with second biochemical recurrence (*miR-628-3p, miR-1193, miR-601, miR-4516, miR-320e,* and *miR-508-3p*) are hypothesized to play a role in conferring radiation

resistance and those miRNAs with low hazard ratios (*miR-598, miR-626*, and *miR-563*) are conferring radiation sensitivity (Table 1).

miR-ID	Hazard Ratio (High vs. Low)	p-value	Confidence Interval	Known vs. novel in PCa	Role (s) in other cancers
hsa-miR-628-3p	6.6	0.0036	1.9-23.5	Known	Yes
hsa-miR-1193	5.0	0.0064	1.6-15.6	Novel	Yes
hsa-miR-601	4.6	0.0037	1.6-12.7	Known	Yes
hsa-miR-4516	3.6	0.0128	1.3-10	Novel	Yes
hsa-miR-320e	3.2	0.0339	1.1-9.6	Known	Yes
hsa-miR-508-3p	3.0	0.0296	1.1-8	Known	Yes
hsa-miR-598	0.3	0.0304	0.1-0.9	Known	Yes
hsa-miR-626	0.3	0.0391	0.1-0.9	Novel	Yes
hsa-miR-563	0.3	0.0228	0.1-0.8	Known	Yes

Table 1. *MiRNAs that correlate with biochemical recurrence after salvage radiation in PCa patients. Hazards ratios were generated using a multivariate Cox regression analysis (lymph node status and Gleason score). Only miRNAs with a significant p-value (<0.05) are included. CI, confidence interval (Bell, 2015).*

Goals

The aim of this project is to functionally characterize biologically relevant miRNAs that play a role in radiation resistance in PCa patients using two approaches.

Aim 1: To functionally characterize the seven miRNAs previously correlated with biochemical failure post-RT in vitro. Previously unstudied miRNAs with the most significant hazard ratios were prioritized in this study and followed up by all remaining miRNAs (see Table 1). The miRNAs were studied through *in vitro* cell culture experiments to determine possible mechanisms in conferring radiation resistance. We hypothesized that miRNAs that correlate with biochemical recurrence post-prostatectomy RT in PCa patients are playing a role in radiation resistance/sensitivity pathways.

Aim 2: To identify novel and validate known radiation resistance pathways in PCa. Sequencing was conducted to determine how small RNA expression differs between radiation-resistant and -sensitive cell lines, yet results are pending. We expect to observe significant differences in miRNA between normal and radiation resistant cell lines; especially those pertaining to DNA damage/repair, cell cycle, cell viability, and apoptosis pathways.

Methods

Cell Culture

DU145, PC-3, and LNCaP prostate cancer cell lines (American Type Culture Collection, Manassas, VA, USA) were maintained in EMEM media (Gibco, Life Technologies) with 10% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin. Cells were maintained at 37 ℃ in 5% CO_2.

MicroRNA Expression

Transfections were performed with *Mir*Vana miRNA mimics (Ambion; Thermo Scientific, Waltham, MA) and Lipofectamine RNAiMAX (Thermo Scientific, Waltham, MA) according to the manufacturer's protocol.

RT-qPCR

RNA was harvested from transfected cell lines and isolated using Trizol reagent (Invitrogen; Thermo Scientific, Waltham, MA) according the manufacturer's protocol. Reverse transcription-quantitative PCR (RT-qPCR) analyses for miRNAs were performed using TaqMan miRNA assays (Applied Biosystems; Thermo Scientific, Waltham, MA) and expression levels were measured using a Real Time Detection System. Relative expression values of *miRNA-508-3p* and *miR-1193* were calculated relative to the small RNA, *RNU6B,* and compared to a negative control miRNA (Ambion; Thermo Scientific, Waltham, MA). *No RT enzyme* and *no RNA* samples were included as experimental controls and all samples were assayed in triplicate.

Clonogenic Assays

DU145 and PC-3 cells were transfected in 60-mm^2 plates. Clonogenics were performed to assess colony formation after irradiation according to published protocol (Franken, 2006). Briefly, after 4 hours, cells were counted and seeded in 6-well dishes in triplicate at low densities corresponding to irradiation dose (75 cells/mL at 0 Gy – 16,000 cells/mL at 10 Gy). Within 24 hours of seeding, plates underwent fractionated X-ray radiation including 0, 2, 4, 6, 8, and 10 Gy doses. Cells were allowed to grow for 2

weeks before being stained with 0.5% crystal violet in 50% methanol solution. Colonies were those consisting of at least 50 cells, which were assessed by eye and with the use of a stereotactic microscope. Colony formation was normalized to the plating efficiency of each miRNA-treated cell line at 0 Gy and assessed by surviving fraction at each irradiation dose.

Cell Proliferation

Cells were transiently transfected and seeded at a density of $1x10^4$ cells per well in a 96-well plate. At 24 hours, cells were fixed with 4% paraformaldehyde. Methylene Blue stain was added for 15 minutes and plates were washed. Methylene Blue extracting solution was added and absorbance was measured at 630 nm.

Cell Apoptosis

DU145 cells were transiently transfected and harvested at 24 hour timepoints up to 96 hours with miRNA or negative miRNA control. Each treatment was stained with Annexin V-FITC antibody and propidium iodide and analyzed via flow cytometry using BD FACSCalibur (BD Biosciences). Relative degree of apoptosis was calibrated using a heat-shocked positive control.

Cell Viability

$1x10^4$ cells were seeded in a 96-well plate in quadruplicate. MTS reagent was added to each well and plates were incubated for 2-4 hours. Absorbance was measured at 490 nm and normalized to 650 nm at 24 hour timepoints. Transfected samples were normalized to a media-only control in quadruplicate and the 24 hour timepoint.

Transwell Migration and Invasion

6.5 mm transwell migration chambers with 8.0 uM pores and Matrigel invasion chambers with 8.0 uM pores (Corning, Corning, NY) were rehydrated in chemoattractant-free media for two hours. 24 hours post-transfection (as described previously), cells were seeded in chemoattractant-free media in chambers ($1x10^5$ cells for migration and $5x10^4$ cells for invasion) above wells containing chemoattractant

(10% Fetal Bovine Serum) in media. 24 hours after seeding, membranes were swabbed with cotton. Membranes were fixed in 100% methanol, stained with 0.5% crystal violet, and washed in water. Membranes were mounted to slides with toluene and cells were counted using ImageJ software (National Institutes of Health, Bethesda, MD).

Scratch Assay

Cells were cultured to 80% confluence in a 6 well dish in triplicate and transfected as described previously. 24 hours post-transfection, cells were treated with mitomycin C (20 uL/well) for 3 hours to inhibit proliferation. The cell monolayer was then scratched and washed with PBS three times. Cells were photographed at 0 and 24 hours. Reduction in scratch volume was assessed using ImageJ software (National Institutes of Health, Bethesda, MD).

γ-H2AX Foci Quantification

DNA damage was assessed via quantification of γ-H2AX foci in irradiated cells. Cells were transfected with miRNA treatments and seeded at $2x10^4$ cells/mL in chambered culture cell slides (Falcon; Thermo Scientific, Waltham, MA). 24 hours after transfection, cells were irradiated at 0, 4, and 6 Gy. Cells were fixed at 5 and 15 minutes and 1, 3, 6, and 24 hours post-irradiation. Cells were incubated in a mouse monoclonal primary antibody to gamma H2A.X (Abcam, Cambridge, MA) and in goat anti-mouse secondary antibody AlexaFluor 488 (Invitrogen; Thermo Scientific, Waltham, MA). DAPI was added to visualize nuclei. Immunofluorescence was assessed and foci were manually counted using an AxioVision 4.8 microscope and software (Carl Zeiss, Inc., Thornwood, NY).

In Silico Target Analysis

MiRNA targets were assessed using at least three of the following *in silico* target databases: TargetScan, microRNA.org, MiRDB, and miRTarBase. All targets from each database were individually assessed for cross-listing in the other databases for greater confidence in target accuracy. Targets cross-

listed in at least three databases were further analyzed for possible functions in cancer and references in previous cancer-related functions.

Ingenuity Pathway Analysis

All miRNAs and corresponding hazard ratios were analyzed via Ingenuity Pathway Analysis (Qiagen, Alameda, CA). MiRNAs were categorized into pathways according to relative proportion of molecular target molecules matching previously established integrated pathways. Signaling pathways were selected for by experimental, highly predicted, and moderately predicted confidence levels and were assessed in all species, all cell lines, and representing all possible mutations. Pathways were further assessed for overlap with mRNA targets predicted by *in silico* databases and by individual miRNA.

RNA Sequencing

Parental cell lines of DU145 and PC-3 were irradiated by X-ray five days a week at 2 Gy/day for nine cycles, totaling 90 Gy to confer radiation resistance. Radiation resistance was assessed by comparing colony formation to radiation sensitive (parental) cell lines. RNA was isolated as described previously and submitted the Genomics Shared Resource Core at the Ohio State University for RNA sequencing. Results are pending.

Results

To characterize the seven miRNAs previously found to be correlated with second biochemical recurrence, a functional screen was performed including a multitude of *in vitro* assays. *MiR-4516* and *miR-601* were excluded from analysis due to existing research already in progress, so the remaining seven miRNAs were categorized into several different stages of analysis. *MiR-628-3p, miR-1193,* and *miR-320e* were prioritized due to their high hazard ratios. High hazard ratios were indicative of high levels of these miRNAs corresponding to worse outcome in patients that experienced second biochemical recurrence (Table 1). Follow-up screens included the remaining miRNAs: *miR-508-3p, miR-598, miR-626*, and *miR-563*. The final stage of screening included an in-depth functional screen of *miR-508-3p* and *miR-1193*, due to promising preliminary results.

MiR-628-3p, miR-1193,* and *miR-320e

Clonogenics were performed as a measure of radiation response in both DU145 and PC-3 after miRNA transfection. Figure 2 shows the response of each miRNA-transfected cell line in response to fractionated radiation and the resulting colony formation relative to a negative miRNA control. *MiR-1193* displayed the greatest sensitivity to radiation over three trials in DU145 and three trials in PC-3.

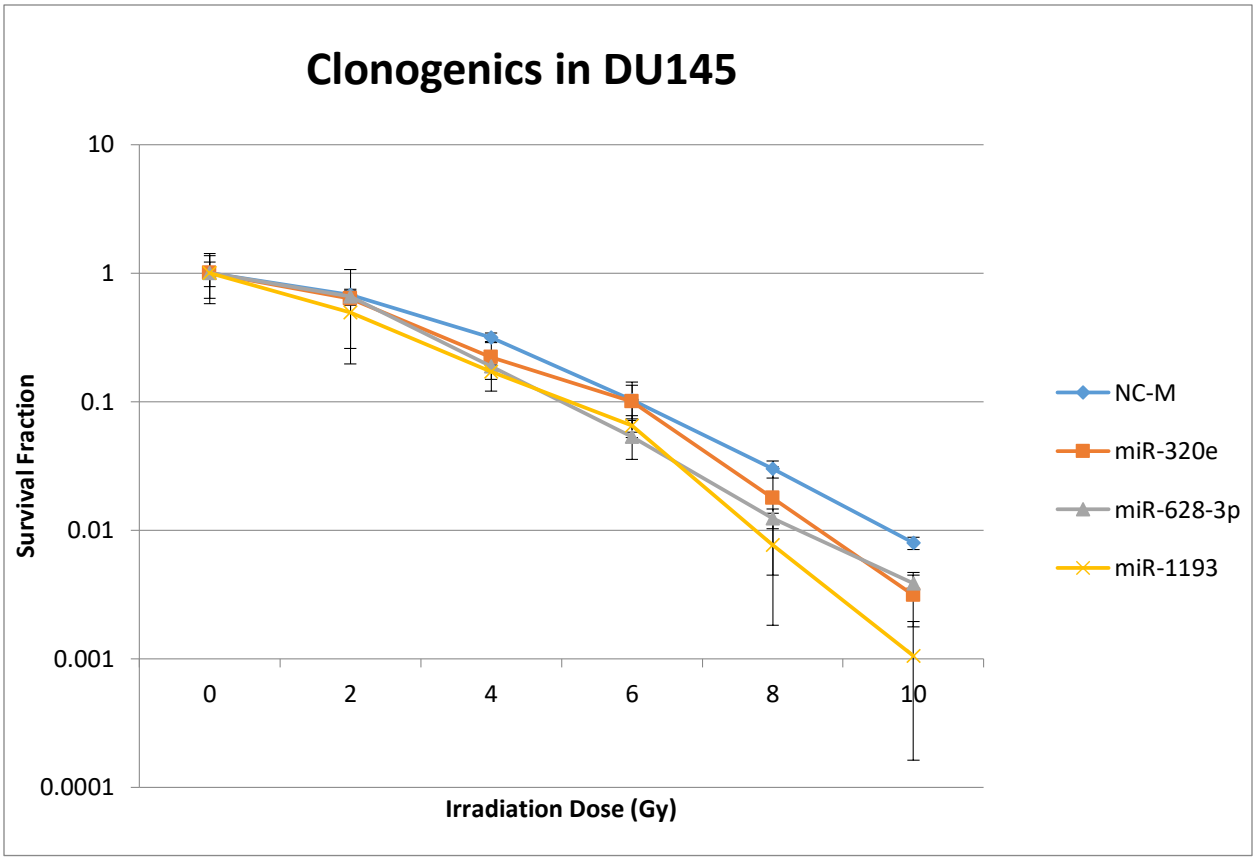

Figure 2A. Clonogenic assay in DU145. Surviving fraction of colonies in response to fractionated X-ray radiation after two weeks. Induced expression of miR-1193 results in increased radiation sensitivity at 10 Gy relative to NC-M according to unpaired t-test (p=0.0068).

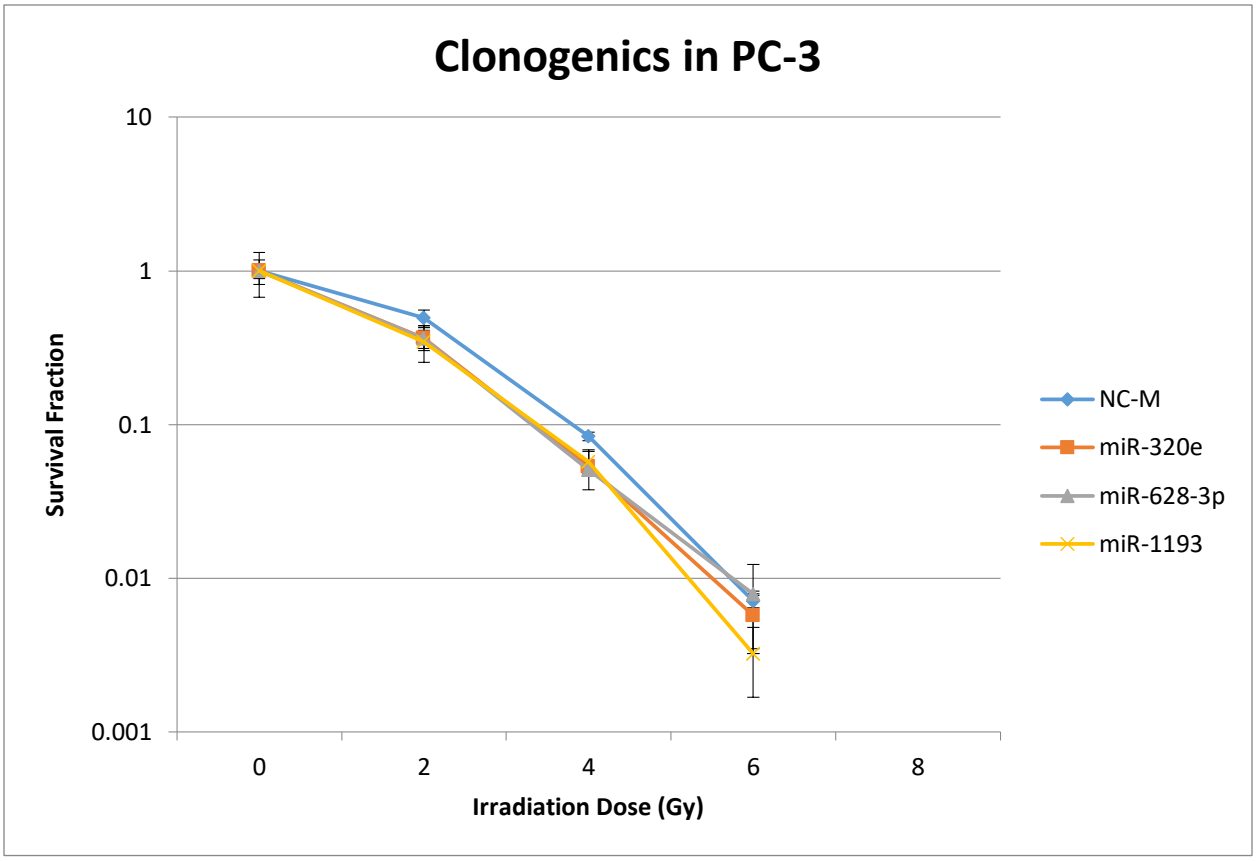

Figure 2B. Clonogenic assay in PC-3. Surviving fraction of colonies in response to fractionated X-ray radiation after two weeks. Induced expression of miR-1193 results in increased radiation sensitivity at 10 Gy relative to NC-M according to unpaired t-test (p=0.0164).

Additional characterization was performed to test the relative effect of each miRNA on cell viability in DU145 and PC-3 over three trials. As seen in Figure 3, *miR-320e, miR-628-3p,* and *miR-1193* all decreased cell viability relative to a negative miRNA control; however, *miR-1193* displayed the most robust effect in both cell lines. As an additional follow-up, the effect of each miRNA on cell proliferation was tested in PC-3, yielding similar results (Figure 4).

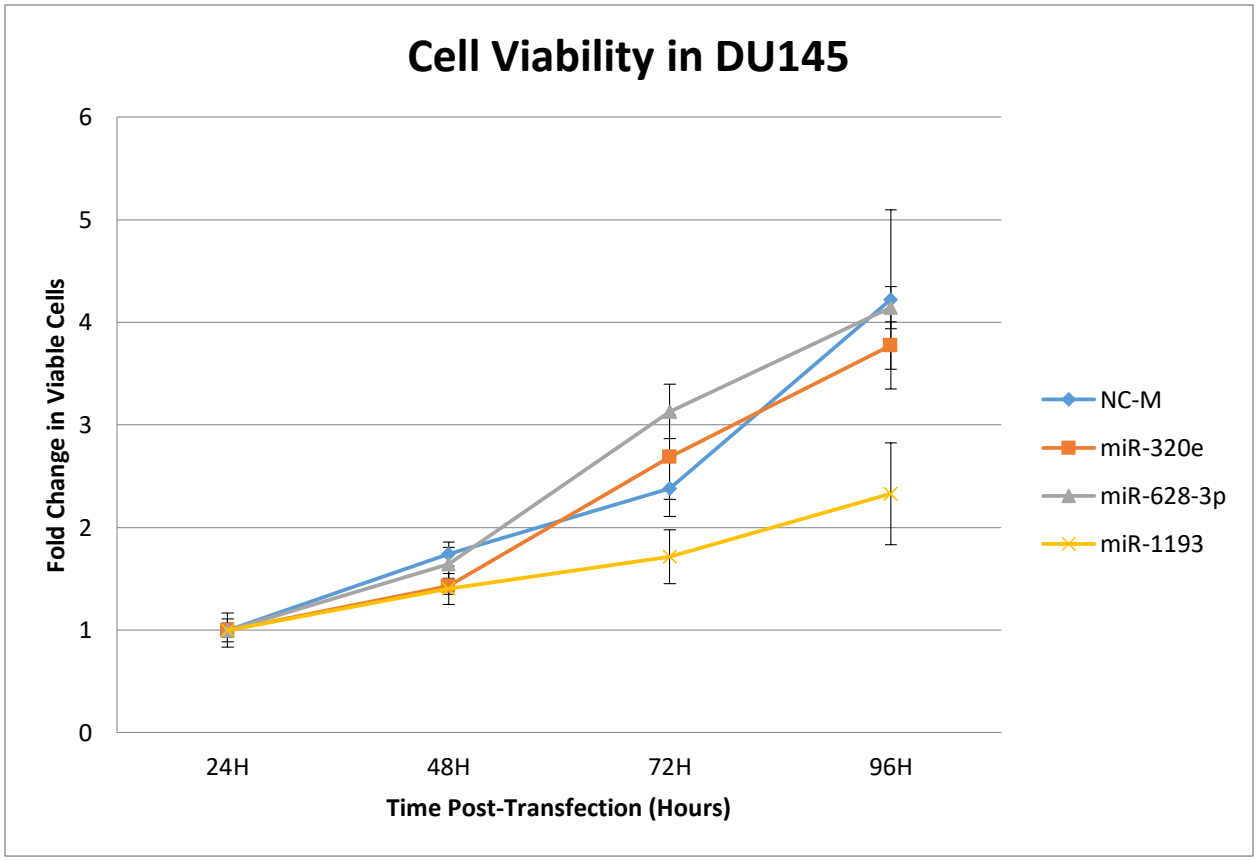

Figure 3A. Assessment of effect of miR-320e, miR-6283p, and miR-1193 on cell viability in DU145 via MTS assay. MiR-1193 displays the greatest reduction in cell viability at 96 hours compared to negative miRNA control according to unpaired t-test (p=0.0309).

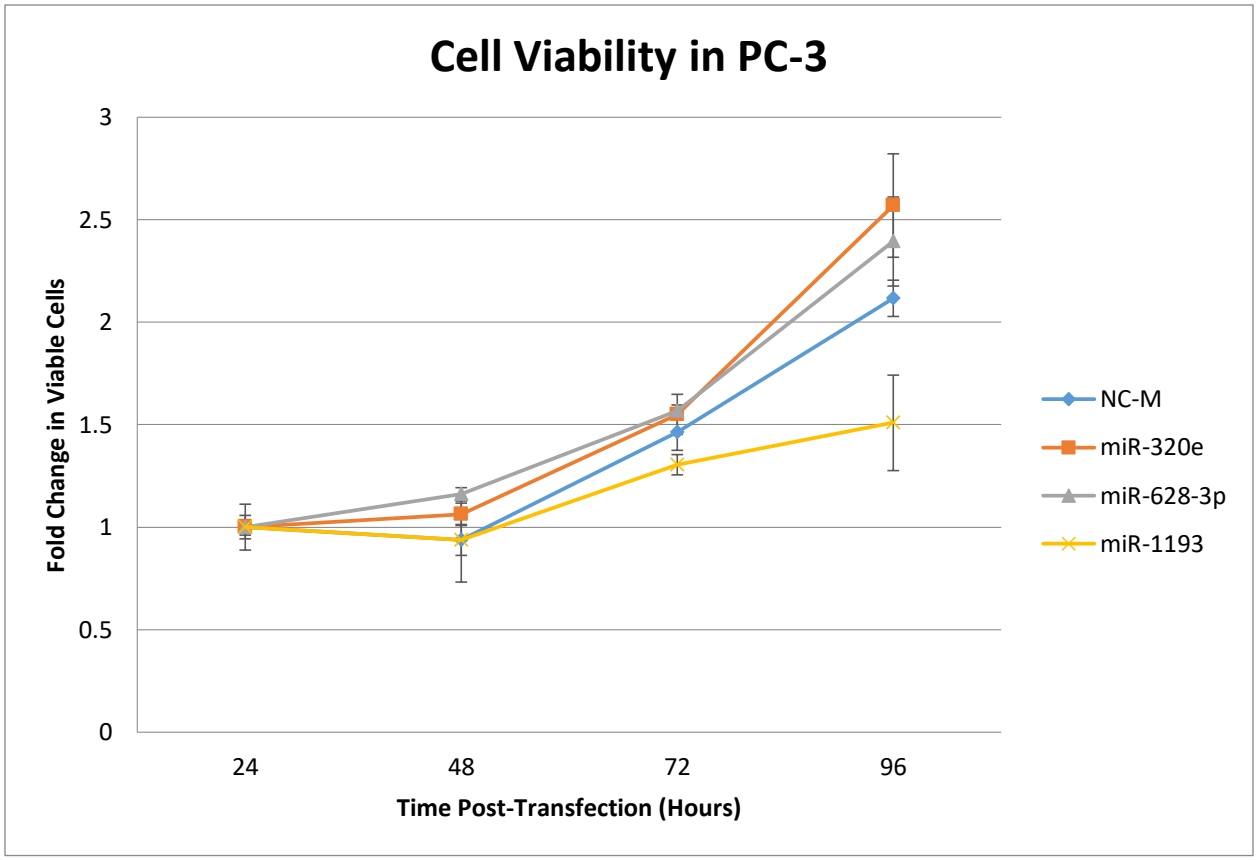

Figure 3B. Assessment of effect of miR-320e, miR-6283p, and miR-1193 on cell viability in PC-3 via MTS assay. MiR-1193 displays the greatest reduction in cell viability at 96 hours compared to negative control according to unpaired t-test (p=0.0038).

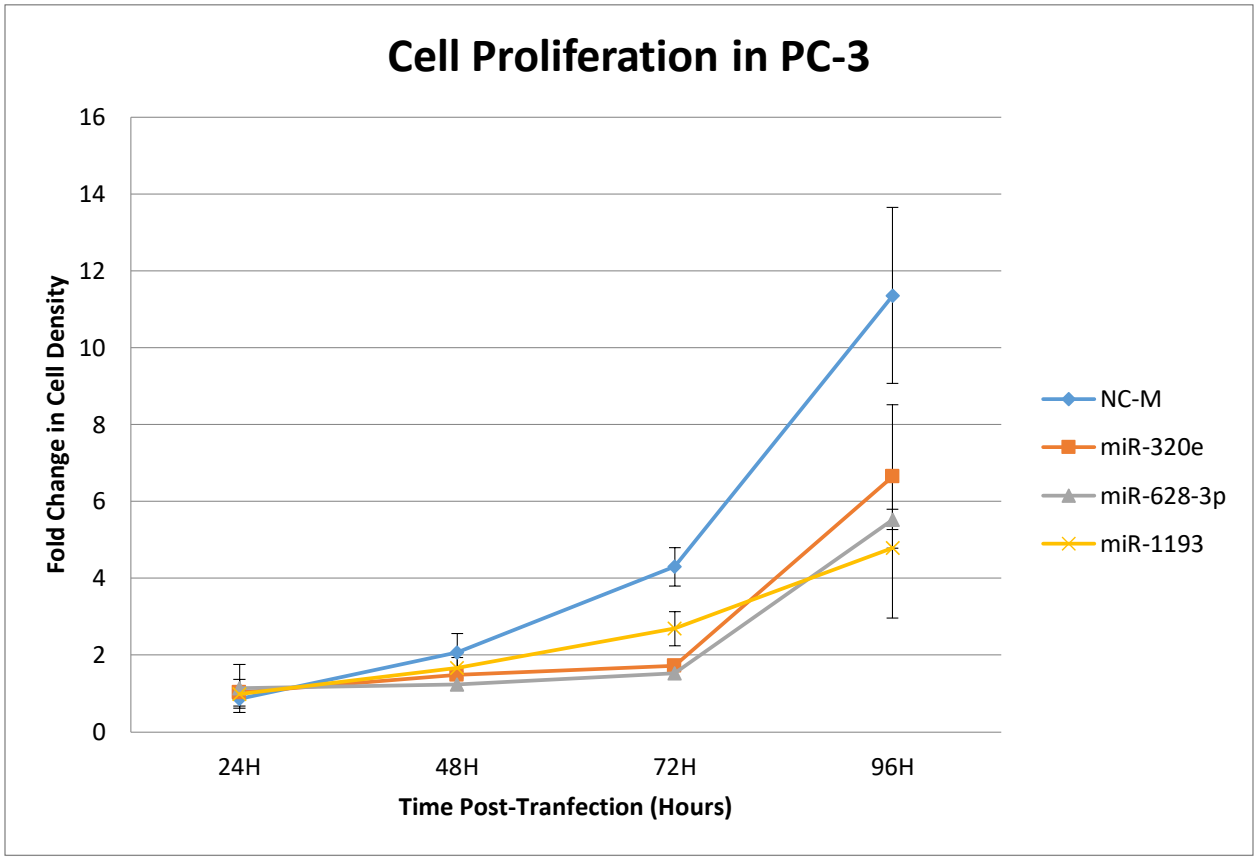

Figure 4. Effect of miR-320e, miR-628-3p, and miR-1193 on cell proliferation in PC-3 via methylene blue assay. MiR-1193 displays the greatest reduction in cell proliferation at 96 hours compared to negative control according to unpaired t-test (p= 0.0178).

To investigate possible mechanisms of the tumor-inhibitory phenotype of these miRNAs, cell apoptosis was evaluated over three trials in DU145. As seen in Figure 5, all miRNAs resulted in a decrease in apoptotic cells, with *miR-1193* having the least amount of apoptotic cells. Therefore, this screen indicated that apoptosis was likely not the mechanism by which colony formation, cell viability, and cell proliferation were being inhibited, and further follow-up was necessary.

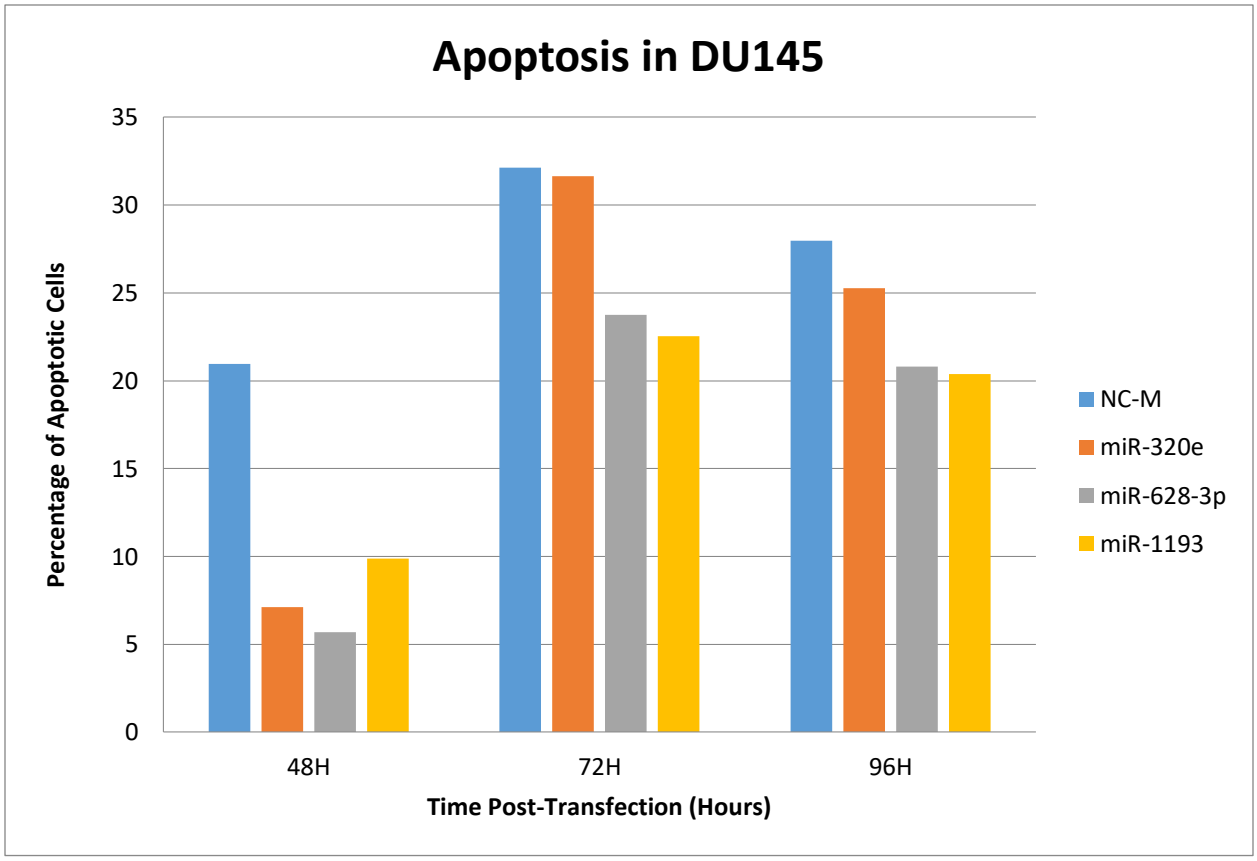

Figure 5. Relative percentage of apoptotic cells in DU145 cells transfected with miR-320e, miR6283p, and miR-1193.

Mir-563, miR-598, miR-626, miR-508-3p,* and *miR-1193

Due to the relatively robust effect of *miR-1193*, it was selected for further analysis. The remaining four miRNAs correlated with second biochemical recurrence were also characterized for a comprehensive analysis. Three additional trials of clonogenic assays in DU145 confirmed the results initially seen with *miR-1193* (see Figure 6). All other miRNAs also displayed sensitivity to radiation; of these, *miR-508-3p* displayed the most significant sensitivity to radiation.

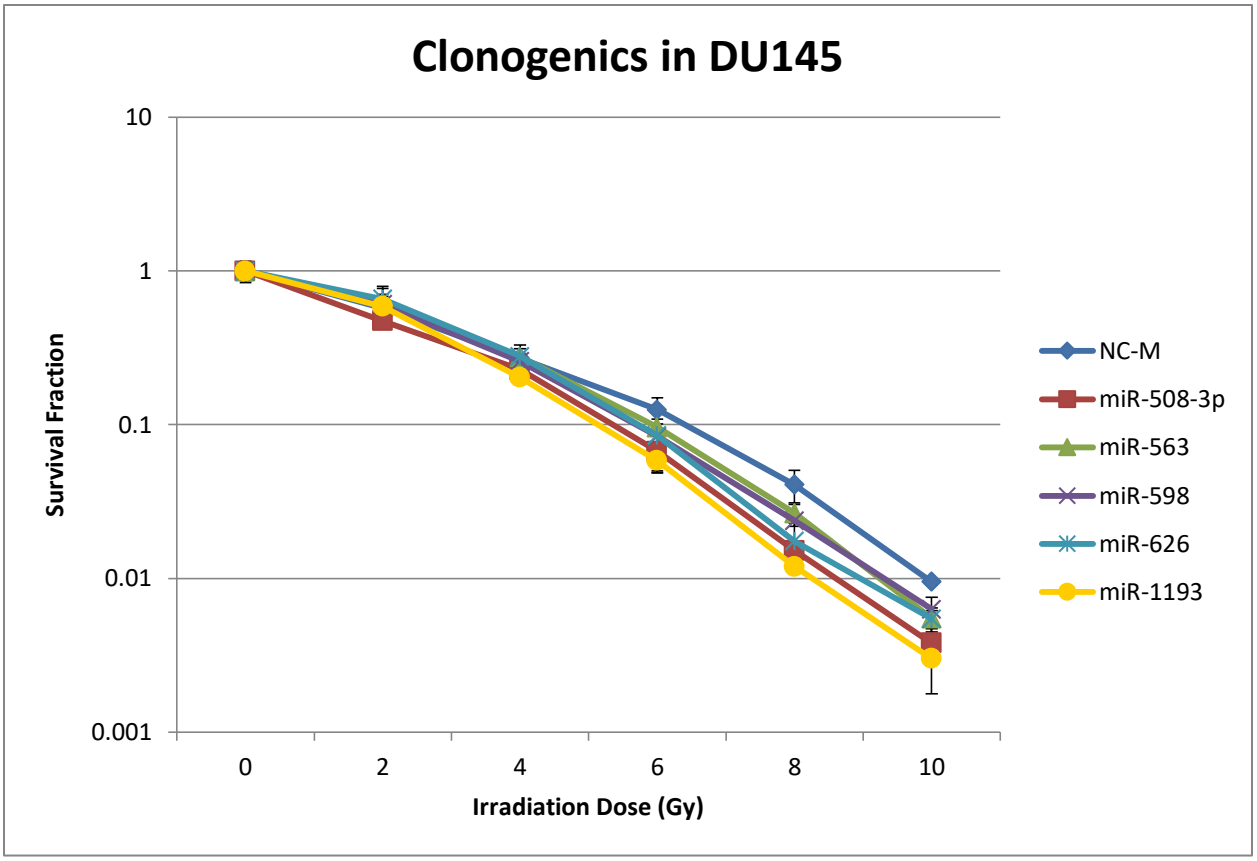

Figure 6A. Surviving fraction of colonies in response to fractionated X-ray radiation after two weeks in DU145. All miRNAs displayed significant sensitivity to radiation; miR-1193 and miR-508-3p displayed the most robust sensitivity to radiation at 10 Gy compared to negative miRNA control according to unpaired t-test (p=0.0065 and p=0.0057, respectively).

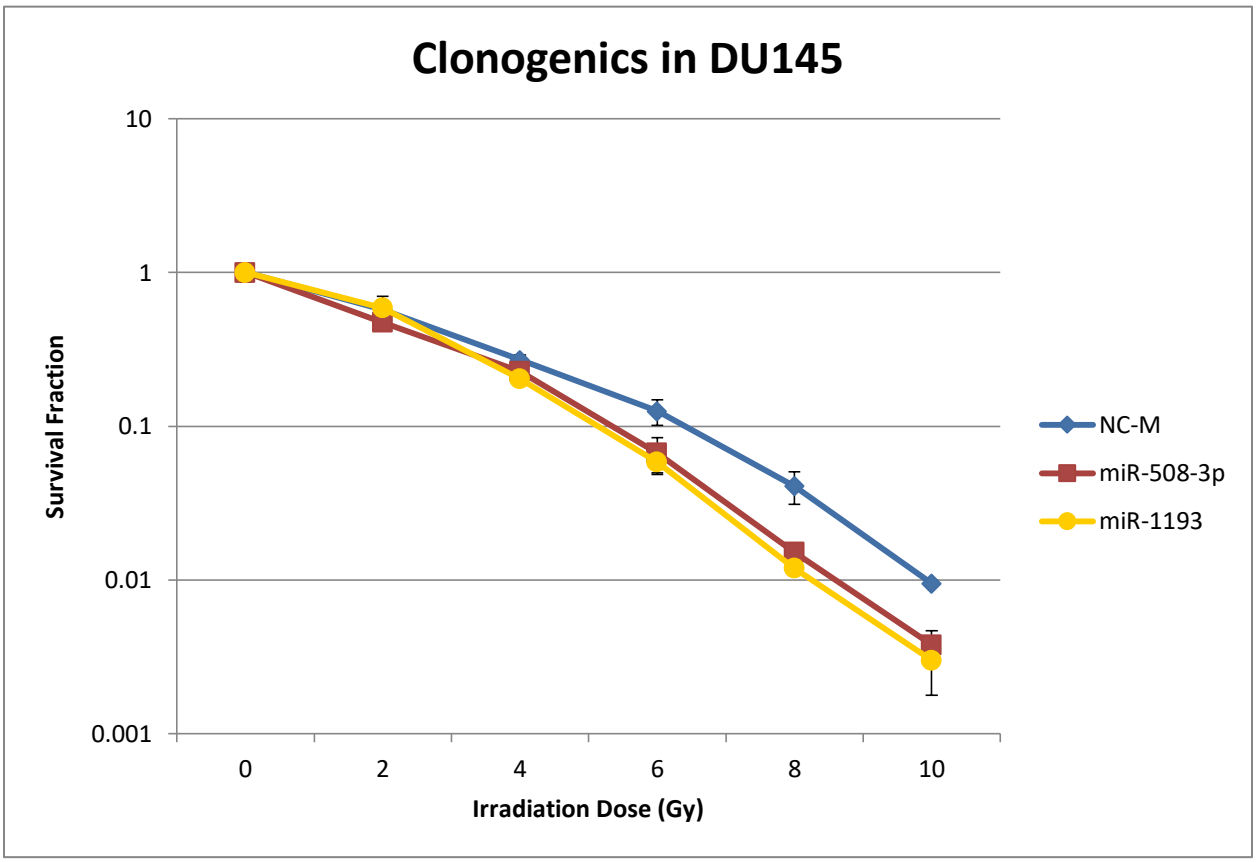

Figure 6B. Figure 6A is replicated with miR-508-3p and miR-1193 separated for clarity.

As an additional screen, an MTS assay was performed on *mir-563, miR-598, miR-626, miR-508-3p,* and *miR-1193* at 96 hours in PC-3 over three trials. Results indicated that all miRNAs decreased cell viability, with *miR-508-3p* having the strongest effect. In addition, *miR-1193* also significantly reduced cell viability (Figure 7).

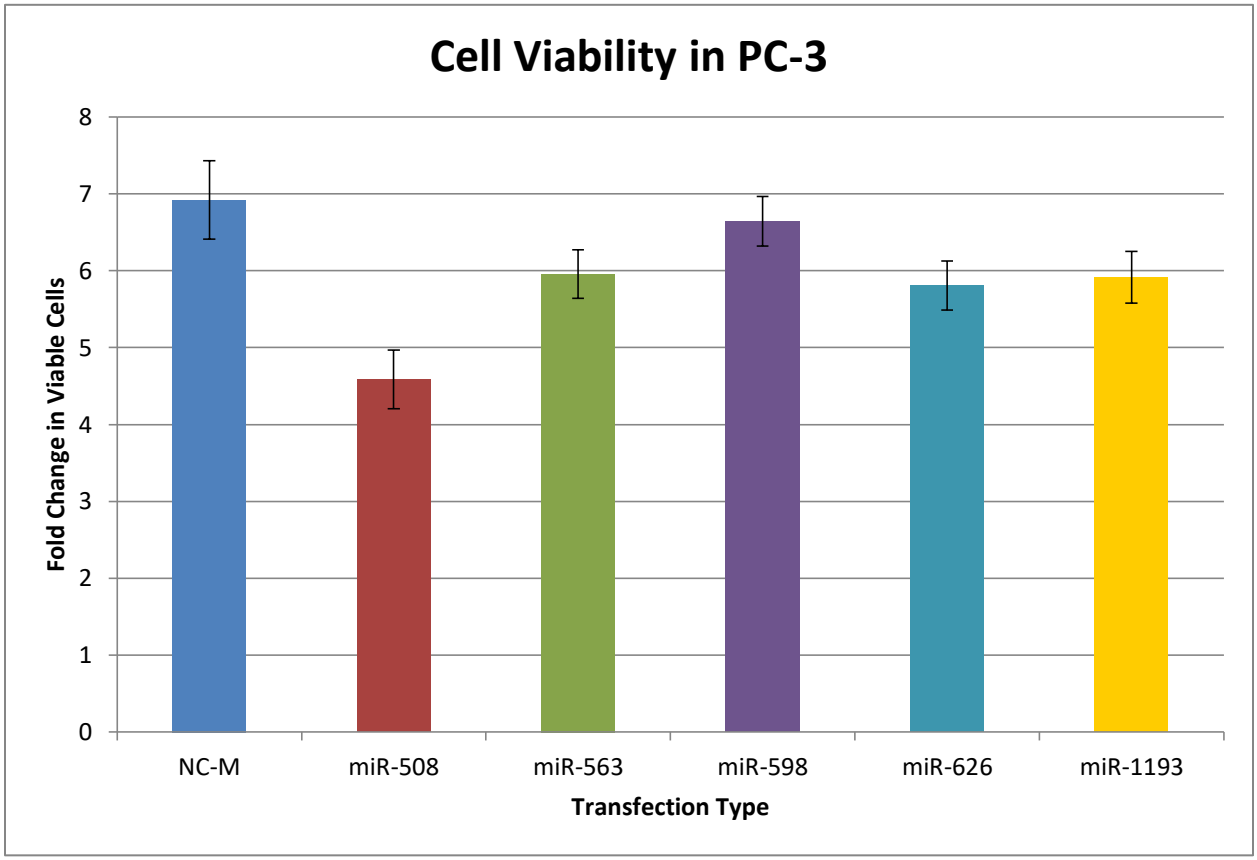

Figure 7. Effects of miRNAs on cell viability in MTS assay in PC-3 at 96H normalized to 24H timepoint. MiR-508-3p and miR-1193 showed a significant reduction in cell viability at 96 hours compared to negative miRNA control according to unpaired t-test (p<.0001 and p=.0061, respectively).

MiR-508-3p **and** ***miR-1193*** **Phenotypic Validation**

MiR-508-3p and *miR-1193* were selected for further follow-up analysis. Transfection efficacy was determined through RT-qPCR in both DU145 and PC-3 cell lines. Figure 8A and 8B indicate that both miRNAs were over-expressed relative to a negative miRNA control at all time points assessed (24-96 hours) in DU145. Similar results were exhibited in PC-3 (data not shown).

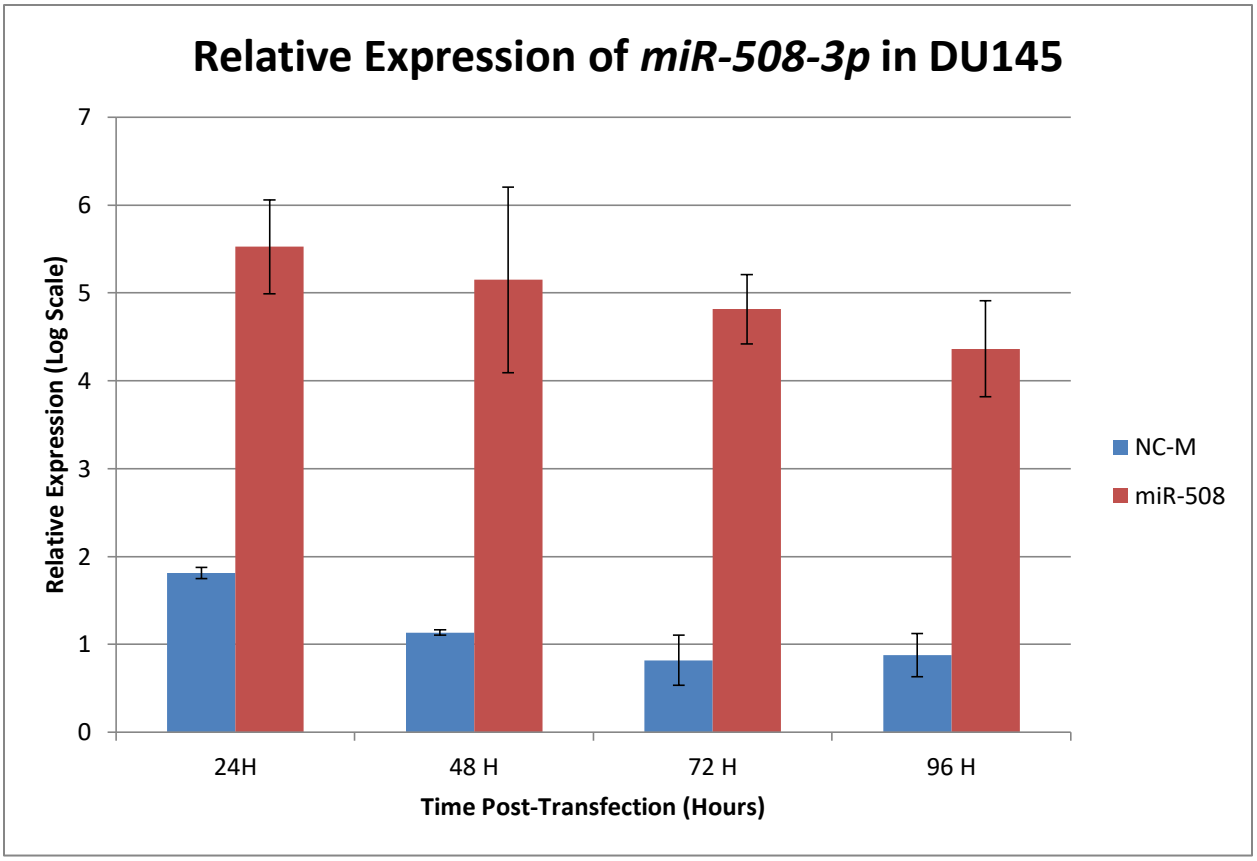

Figure 8A. Relative expression of miR-508-3p relative to a miRNA negative control (log scale) in DU145; similar results seen in PC-3 (data not shown).

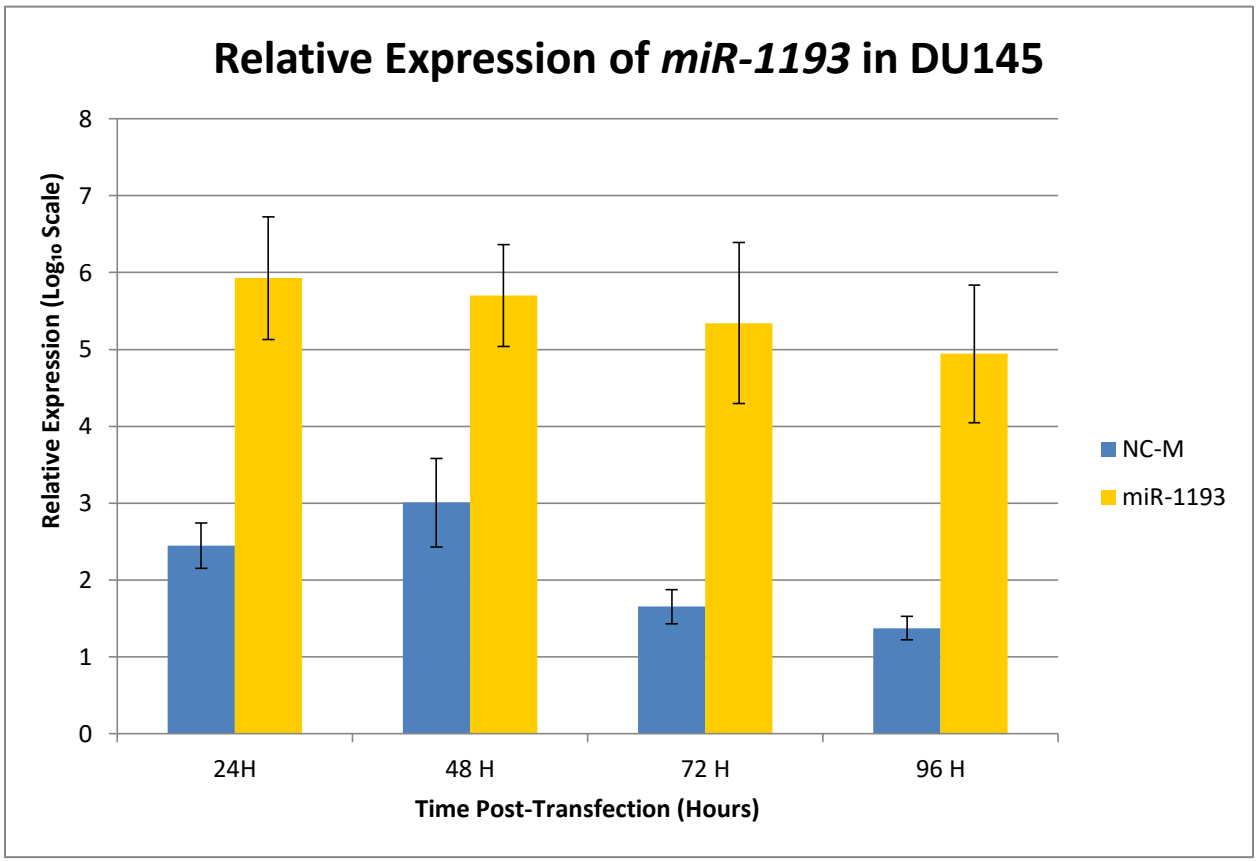

Figure 8B. Relative expression of miR-1193 relative to a miRNA negative control (log scale) in DU145; similar results seen in PC-3 (data not shown).

Endogenous expression of both miRNAs relative to a small RNA control, *RNU6B*, was also determined through RT-qPCR in DU145, PC-3, and LNCaP cell lines (Figure 9). Endogenous expression of both *miR-508-3p* and *miR-1193* was very low in all three cell lines at all passages.

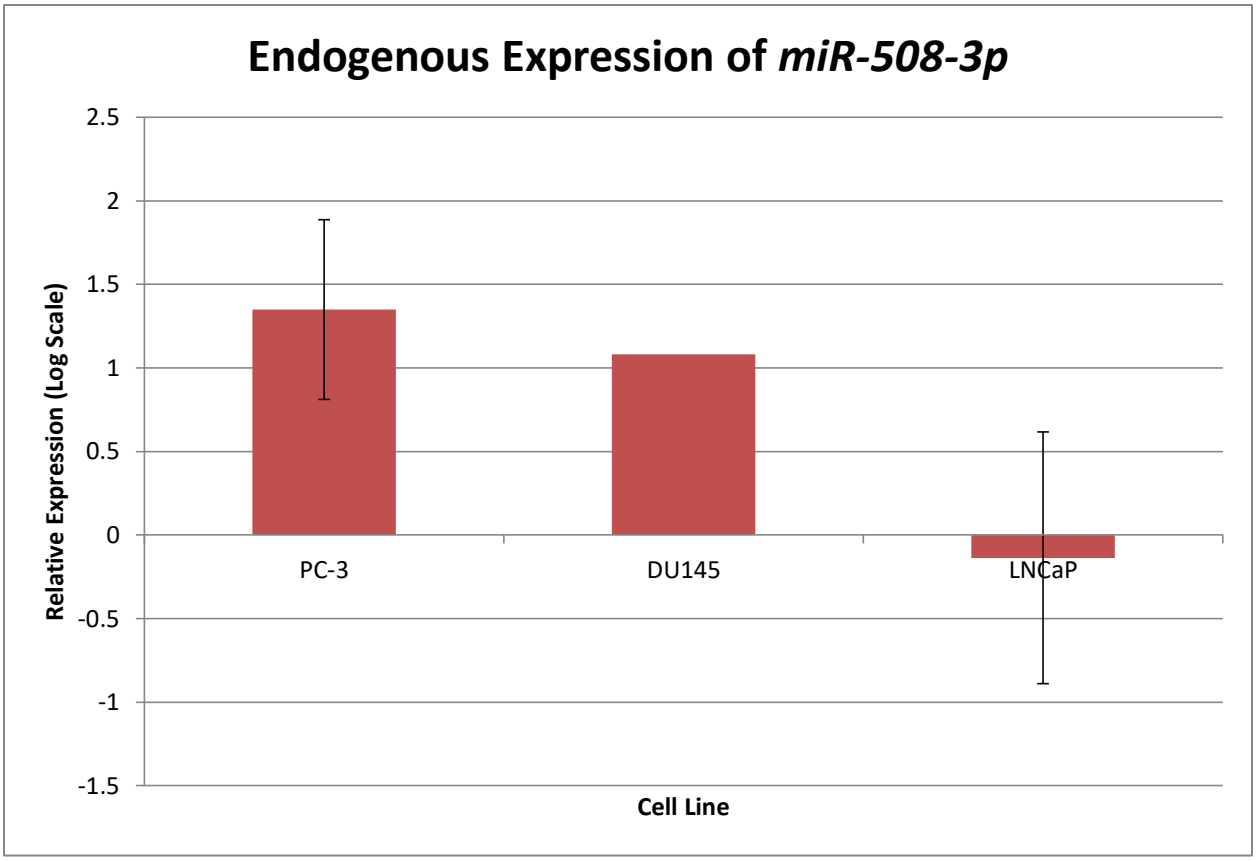

Figure 9A. Endogenous expression miR-508-3p relative to small RNA RNU6B in three passages each of PC-3, DU145, and LNCaP.

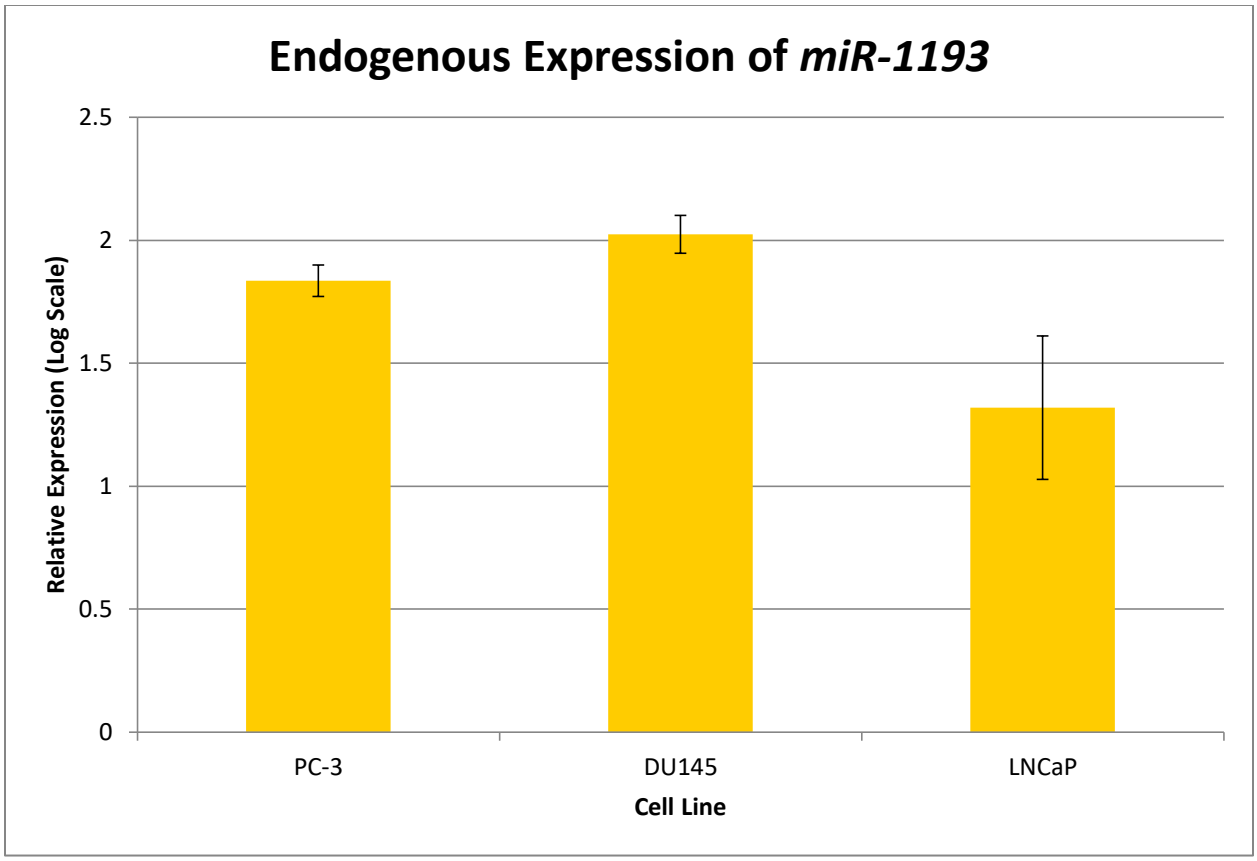

Figure 9B. Endogenous expression miR-1193 relative to small RNA RNU6B in three passages each of PC-3, DU145, and LNCaP.

Further characterization of *miR-508-3p* and *miR-1193* was performed by assessing the relative degree of migration and invasion of DU145 cells transfected with each miRNA. Migration and invasion were initially assessed using transwell chamber inserts over three trials, but data were not significant (shown in Figure 10A and 10B).

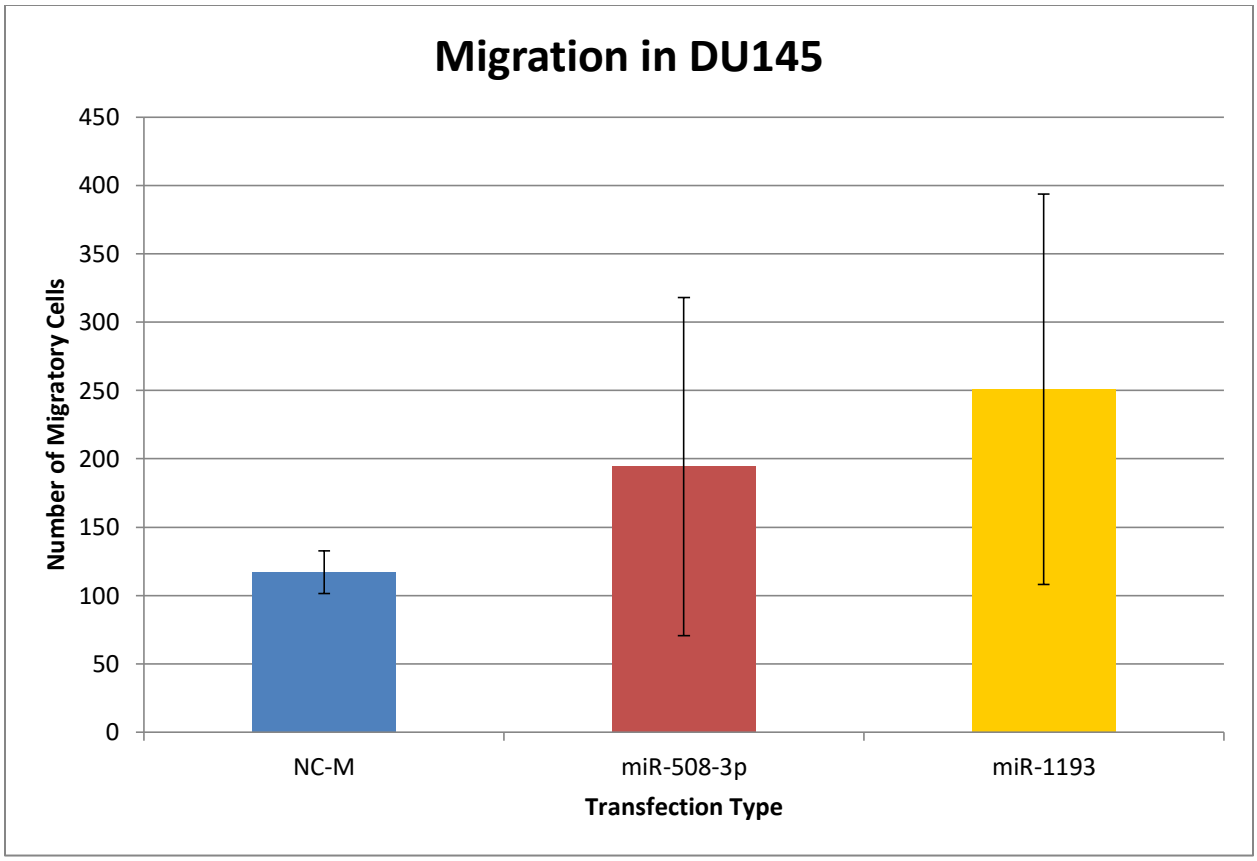

Figure 10A. Number of migratory cells in miR-508-3p and miR-1193 treated cells compared to negative miRNA control in DU145. Data were not significant.

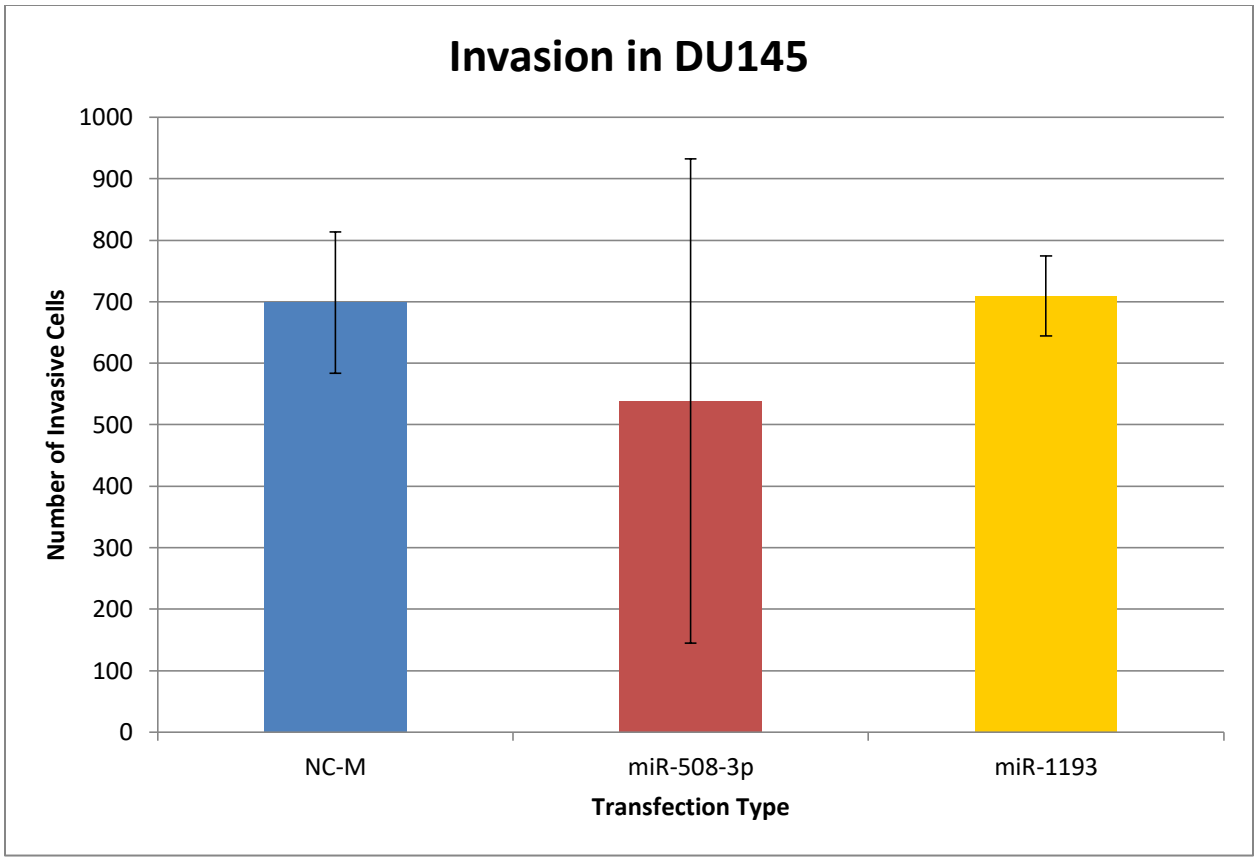

Figure 10B. Number of invasive cells in miR-508-3p and miR-1193 treated cells compared to negative miRNA control in DU145. Data were not significant.

Follow-up characterization was performed using the scratch assay method of assessing migration as seen in Figure 11. Two trials of the scratch assay validated the original conclusion that relative degrees of migration among *miR-508-3p* and *miR-1193* compared to a negative control were not significant.

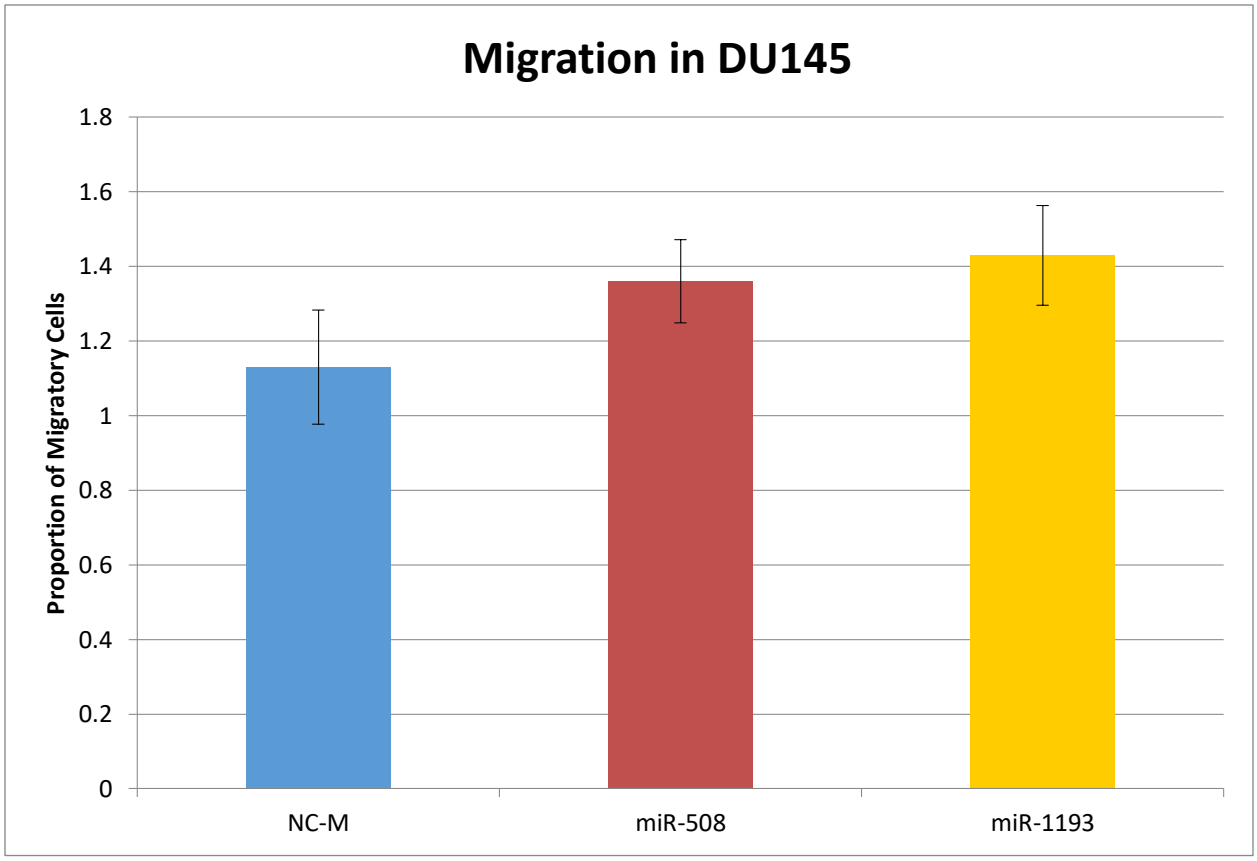

Figure 11. Proportion of migratory cells in DU145. Neither miRNA displayed significant migration according to unpaired t-test (miR-508-3p: p= 0.2329; miR-1193: 0.1692).

A possible mechanism of increased sensitivity to radiation in *miR-508-3p* and *miR-1193* transfected cells was assessed through the quantification of γ-H2AX foci as a representation of the conferral of DNA DSBs by each miRNA. The H2AX histone becomes phosphorylated in response to DNA DSBs, mainly via the ATM protein kinase as part of the PI3K (Phosphatidylinositol 3-Kinase) family cascade (Paull, 2000). Quantification was only assessed over one trial in DU145, and therefore should not be interpreted as conclusive, but merely as a trend to investigate in future directions. Figure 12 indicates that *miR-508-3p* and *miR-1193* resulted in a 75% and 66% average increase in γ-H2AX foci after irradiation compared to a negative control, respectively. While variability prevented these data from

reaching statistical significance, they suggest a possible trend of radiation-induced DNA damage in *miR-508-3p* and *miR-1193* transfected cells to investigate in future trials.

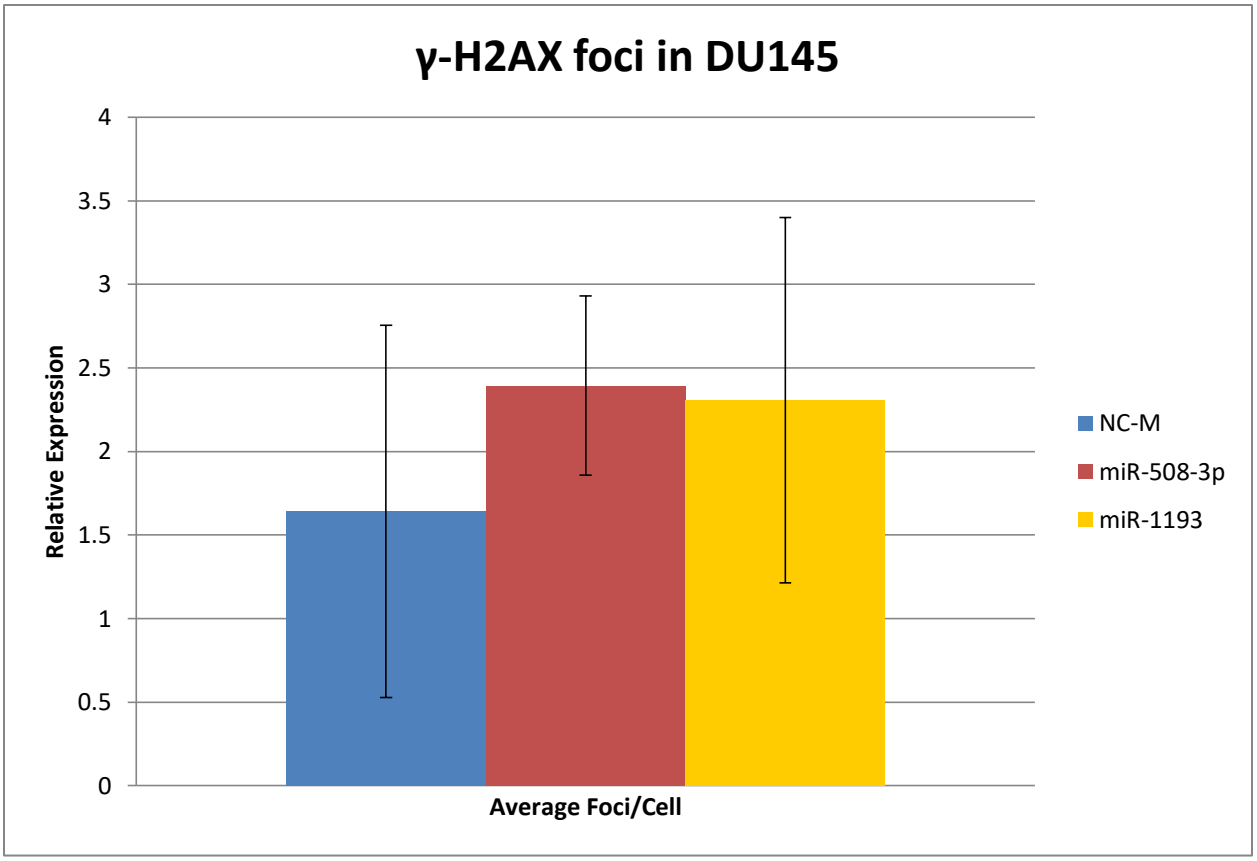

Figure 12. Relative expression of γ-H2AX foci in miR-508-3p and miR-1193 transfected cells in DU145 15 minutes post-irradiation. Irradiation was completed at 4 Gy and normalized to 0 Gy.

Discussion

PCa is one of the most common cancers in the United States, currently affecting nearly 3 million American men. About 1 in 39 of these men will die from the disease, and all will suffer from a reduced quality of life. Aside from the repercussions of the disease itself, many of the treatment options cause undesirable side effects (American Cancer Society, 2017). Moreover, treatment may not be aggressive enough for some patients with advanced disease, while treatment may be completely unnecessary in others. However, current clinical standards lack specificity, and there is currently no reliable method of discriminating between indolent and aggressive disease. In addition, there are no clinical biomarkers currently utilized to predict response to treatment to PCa, and no miRNA biomarkers. Further research is required to provide clarification for physicians and patients on potential therapeutic efficacy. Thus, there is significant potential for molecular markers to further refine and enhance clinical decision making.

Previous research has indicated the potential of miRNAs to provide insight as a possible prognostic or predictive biomarker. In this study, seven miRNAs previously correlated with second biochemical recurrence in PCa patients were screened *in vitro.* All seven miRNAs, including *miR-628-3p, miR-1193, miR-320e, miR-508-3p, miR-598, miR-626,* and *miR-563,* were shown to have potential by demonstrating sensitivity to radiation treatment and decreasing cell viability. However, of these seven miRNAs, *miR-508-3p* and *miR-1193* consistently showed the strongest effect. Specifically, cell lines expressing *miR-508-3p* and *miR-1193* sensitized cells the most to radiation treatment ($p=0.0057$ and $p=0.0065$, respectively; Figure 2) and displayed the greatest reduction in cell viability ($p<.0001$ and $p=.0061$, respectively; Figure 3).

The radiation sensitizing effect displayed in clonogenic assays as well as the decreased cell viability and proliferation displayed via MTS and methylene blue assays suggest a tumor inhibitory phenotype for both miRNAs. Follow-up screening of *miR-508-3p* and *miR-1193* validated this phenotype;

neither miRNA displayed significant evidence of an aggressive migratory phenotype by transwell migration assay or scratch assay (Figures 10A and 11). Further, neither *miR-508-3p* nor *miR-1193* displayed an invasive phenotype by transwell invasion assay (Figure 11). However, assessment of *miR-1193* via Annexin V/PI staining did not indicate a significant increase in apoptosis (Figure 5), so the mechanisms of tumor suppression remain unclear. Quantification of γ-H2AX foci in *miR-508-3p* and *miR-1193* transfected cells suggest that these miRNAs may be contributing to increased levels of DNA damage via DSBs in cancer cells, but results are too preliminary to be conclusive.

Characterization of *miR-508-3p* and *miR-1193* remains complex beyond *in vitro* validation. Results from our original study, utilizing a cohort of patients with PCa who underwent surgery followed by salvage RT, suggested that higher levels of *miR-508-3p* and *miR-1193* are associated with worse outcome and/or lack of response to salvage RT (Table 1) (Bell, 2015). The high hazard ratios associated with these miRNAs (3.0 for *miR-508-3p* and 5.0 for *miR-1193)* does not suggest a tumor-inhibitory phenotype for either of these miRNAs. However, there are several possible interpretations of these results. First, biomarkers are not necessarily representative of biological function. FFPE samples from patients were taken from prostate tumor tissue before radiation treatment was administered; therefore miRNA expression is not a result of treatment. Instead, miRNA expression may be representative of either oncogenesis or regulation of cancer growth. In this context, high levels of *miR-508-3p* and *miR-1193* may suggest an inherent mechanism of inhibiting tumor progression. In this scenario, a tumor-inhibitory phenotype of *miR-508-3p* and *miR-1193* may be acceptable.

Other studies have previously identified *miR-508-3p* as a tumor suppressor, such as by inhibiting cell proliferation, inducing apoptosis, and inhibiting cell migration in renal cell carcinoma (Zhai, 2012). According to the miRGator database, which gathers 73 deep sequencing datasets on human samples from GEO, SRA, and TCGA archives, *miR-508-3p* displayed negligible expression in prostate adenocarcinoma compared to epithelial tissue (miRGator, 2017). In addition, *miR-508-3p* was not highly

expressed in cancerous tissue relative to epithelial tissue in our patient cohort (Bell, 2015). While this suggests that the role of *miR-508-3p* is not likely to be tumorigenesis, further follow-up in additional patient cohorts is necessary. *MiR-1193* is relatively unknown, but has recently been shown to suppress proliferation and invasion and target the oncogene *IGF2BP2* in breast cancer (Li, 2016). The miRGator database suggests that *miR-1193* is expressed at a low level in prostate adenocarcinoma, warranting similar conclusions to those of *miR-508-3p.*

Further understanding of these miRNAs requires the validation of their status as biomarkers. *MiR-508-3p* and *miR-1193* expression was analyzed within one treatment group only, i.e. patients that received salvage radiation therapy after surgery. Our past study suggested that *miR-508-3p* and *miR-1193* are predictive biomarkers (predictive of response to salvage RT), but because only one treatment group was analyzed, it is possible that these miRNAs may only be prognostic and rather are representative of overall patient outcome. However, as miRNA expression was assessed before treatment, their role in affecting response to treatment cannot be assumed from expression patterns alone. Analysis of these miRNAs in additional cohorts of patients receiving other therapeutic interventions, as well as validation in another salvage RT cohort, could provide insight into the role of these miRNAs as biomarkers.

Interpretations of these miRNAs may also be limited due to experimental parameters. Patient samples were collected from a small subset of the original cohort (n=43), corresponding to those patients that experienced second biochemical recurrence (n=19). Limitations in sample size therefore could prevent generalized interpretations of the role of these miRNAs from patient data alone. Further, *in vitro* and *in vivo* experiments are inherently limited in their application due to the relatively isolated context of cell lines and mouse populations compared to the human body. Therefore, both *in vitro* and clinical tissue samples represent potential limitations in accurately interpreting data.

Studies of other miRNAs have shown similar levels of complexity. The *let-7* family of miRNAs has also been studied in the context of radiation treatment due to its targeting of *KRAS,* a radioprotective pathway. However, *let-7* has been shown to be downregulated in lung cancer cell lines yet upregulated in glioma cell lines, which indicates the role of other factors in regulating miRNAs and their response to radiation (Metheetrairut, 2013). Despite their complexity, the dynamic expression patterns of miRNAs suggest their promise as clinical targets. Several miRNA targets are currently undergoing various stages of clinical trials; preclinical trials have been completed regarding *miR-10b* in glioblastoma, *miR-155* in hematological malignancies, and *miR-15* in myocardial infarction, among others (Christopher, 2017). In addition, *miR-16*, which has been implicated as a tumor suppressor in malignant pleural mesothelioma and non-small cell lung cancer, has been developed as a therapeutic strategy as a miRNA mimic targeted via anti-epidermal growth factor receptor (EGFR) nanocells. Phase I trial MesomiR 1 has indicated promising results, including tolerance to treatment in a cohort of 6 patients, as well as therapeutic efficacy in one patient (Kao, 2015). However, at least one clinical trial has been stopped prematurely; Phase I of MRX34 was testing a *miR-34* mimic in primary liver cancer and other advanced solid tumors and hematological malignancies, but the study was halted after the observation of severe immune-related adverse events in enrolled subjects (Business Wire, 2016).

Preliminary results in preclinical and clinical trials of miRNA therapies suggest the importance of additional miRNA therapy research. Although mechanisms of *miR-508-3p* and *miR-1193* are not fully elucidated in the present study, follow-up studies are warranted and may provide a more developed comprehension of mechanisms of cancer development and progression as well as radiation response in PCa. Further, the selected miRNAs provide insight into factors contributing to second biochemical recurrence and additional follow-up could help identify the potential of these miRNAs as truly predictive biomarkers.

In silico target searches of *miR-508-3p* and *miR-1193* also provide insight to the pathways influenced by these miRNAs and offer guidance as to future follow up experiments. Targets were identified and validated in at least three target databases including TargetScan, microRNA.org, MiRDB, and miRTarBase; results are shown in Figures 13 and 14. According to these results, *miR-508-3p* appears to target *PPP1R15B,* or protein phosphatase 1, regulatory subunit 15B. *PPP1R15B* has been previously linked to ERα-positive breast cancer as a survival factor, where high expression levels were associated with poor outcome (Shahmoradgoli, 2013). *MiR-508-3p* also appears to target *SRSF1,* or serine/arginine-rich splicing factor 1. Overexpression of this gene in small cell lung cancer was also associated with poor survival in patient cohorts (Anczuków, 2012). Additionally, *miR-1193* is predicted to target *LRRC1,* or leucine rich repeat containing 1. *LRRC1* has been identified as an oncogene in other cancers; specifically, this gene was shown to promote growth and colony formation of hepatocellular carcinoma cells *in vitro* and *in vivo* (Li, 2013). These data correlates well with the tumor-suppressive phenotypes of *miR-508-3p* and *miR-1193* established *in vitro.*

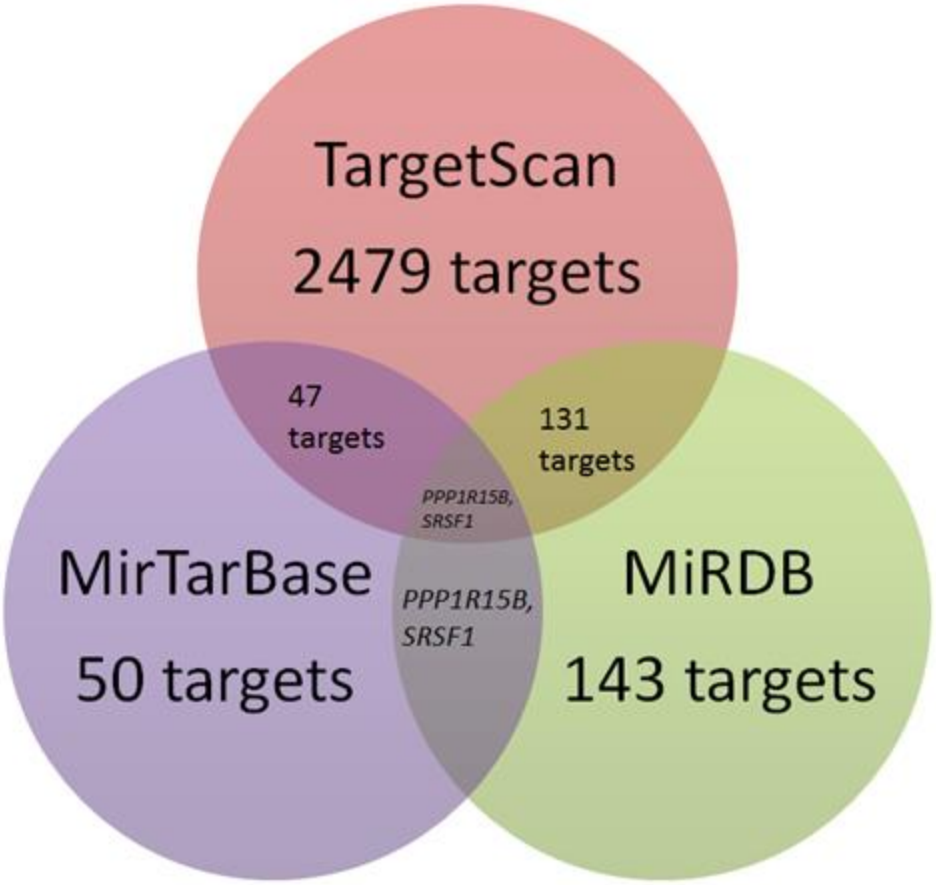

Figure 13. In silico targets of miR-508-3p via TargetScan, miRTarBase, and MiRDB.

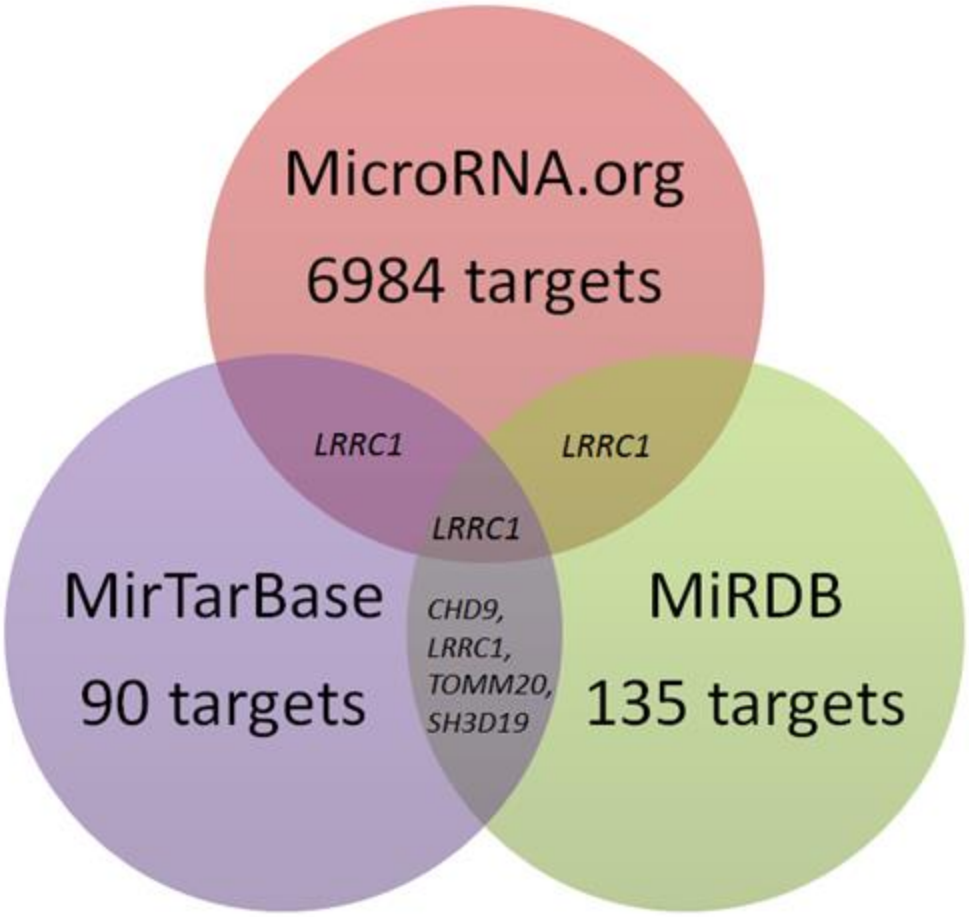

Figure 14. In silico targets of miR-1193 via microRNA.org, miRTarBase, and MiRDB.

Validation of these targets requires *in vitro* follow-up via Western Blot and/or luciferase assay to confirm downregulation of *PPP1R15B* and *SRSF1* by *miR-508-3p* and of *LRRC1* by *miR-1193.* Understanding the functions of these genes can provide insight to the mechanisms of these miRNAs. *PPP1R15B* has been shown to function under conditions of cell stress, where deficiency causes impairment of G1/S cell cycle transition, and that downregulation of this gene could increase apoptosis (Shahmoradgoli, 2013). Further, splicing factor *SRSF1* is known to regulate constitutive and alternative splicing and is frequently upregulated in cancer. It has been shown to initiate cell transformation by increasing cell proliferation and delayed apoptosis in breast cancer (Anczuków, 2012). Interestingly,

SRSF1 has also been shown to regulate the PI3K/AKT and MAPK pathways; kinases of the PI3K family are implicated in radiation response by driving a phosphorylation cascade in response to DNA DSBs (Jiang, 2016; Liu, 2017). Knock down of oncogenic *SRSF1* by miRNA inhibition could therefore represent a mechanism of increased radiation sensitivity. In addition, *LRRC1,* proposed to be targeted by *miR-1193,* also has been suggested to promote cell transformation towards malignancy (Li, 2013).

Investigation of possible targets was further assessed via Ingenuity Pathway Analysis (Qiagen, Alameda, CA). All nine miRNAs correlated with second biochemical recurrence were analyzed for coherence within established signaling pathways, as determined by the percentage of mRNA targets that overlap with the molecules of a specific network. *MiR-1193* was excluded from analysis due to lack of established signaling information. The top 20 networks for the remaining eight miRNAs are displayed in Figure 15. Results indicate that the top pathway is Molecular Mechanisms of Cancer (-log (p-value) = 41.365) shown in Figure 16. Although *miR-1193* was not available for analysis, the network pathway for *miR-508-3p* is shown in Figure 17. While *PPP1R15B* and *SRSF1,* previously identified by *in silico* searches, were not identified within this network, several alternative targets can be identified. Among other targets, *mir-508-3p* is shown to directly target *PIK3C2G*, a gene involved in cell proliferation, oncogenic cell transformation, cell survival, and cell migration (NCBI). *PIK3C2G* is also part of the PI3 kinase family, which, as previously mentioned, is implicated in radiation response by the recruitment of DNA damage repair proteins (Liu, 2017).

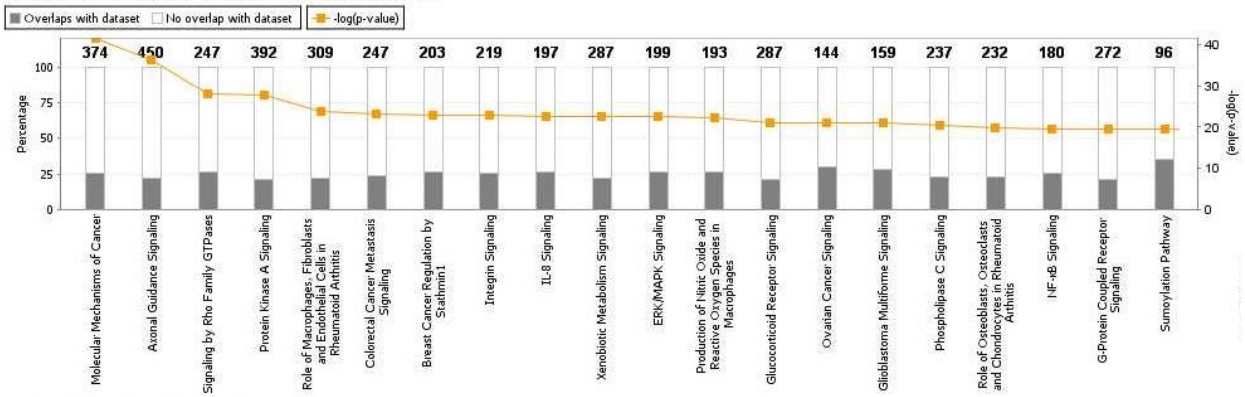

Figure 15. Top twenty pathways correlating with miRNAs linked to second biochemical recurrence in PCa. Percentage of targets that overlap between the miRNA and within the established network is visible on the left y-axis and the (-log (p-value)) is displayed via the trend line and the right y-axis.

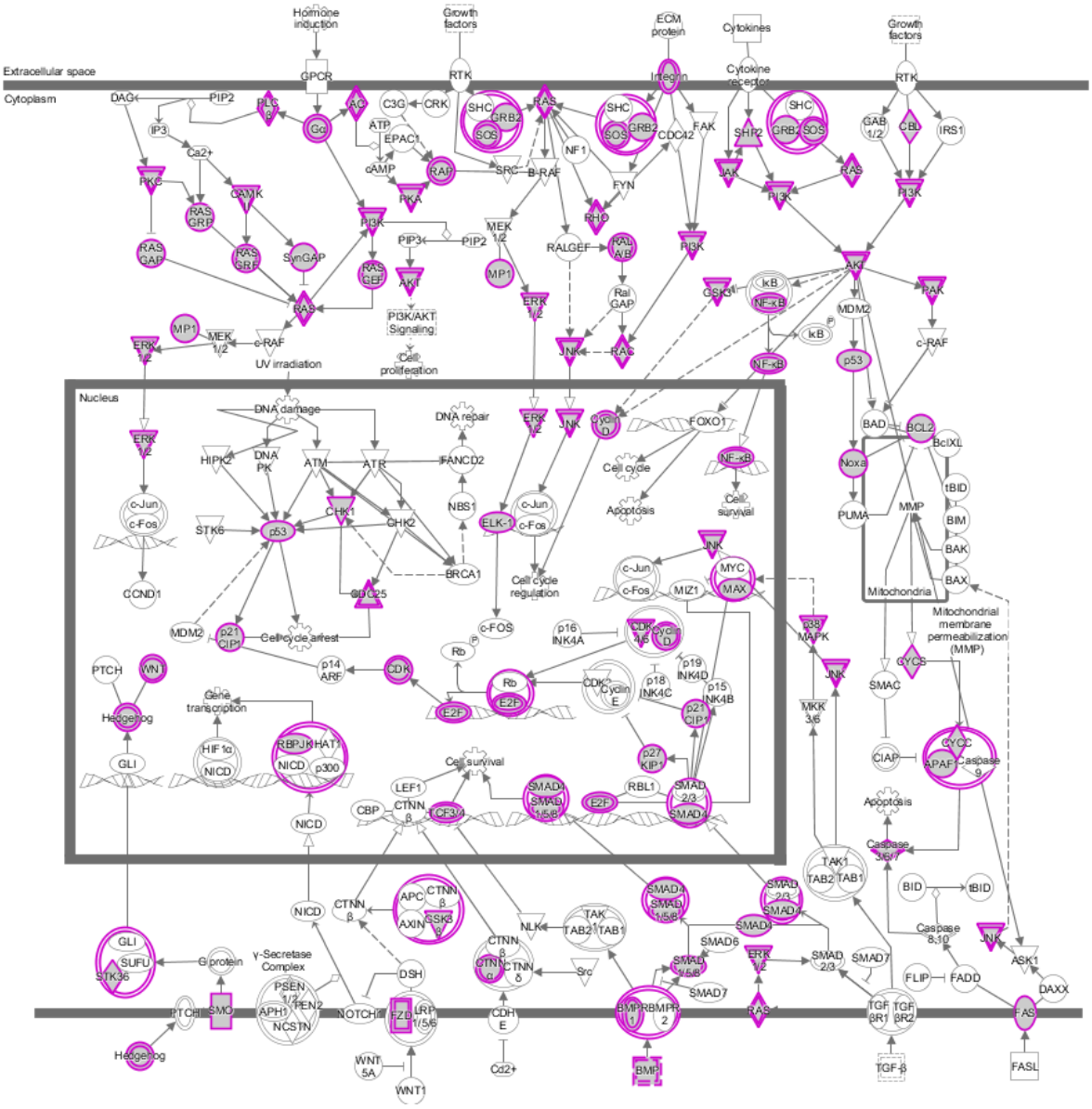

Figure 16. Molecular Mechanisms of Cancer pathway via IPA analysis. MiRNAs associated with second biochemical recurrence and their corresponding hazard ratios were assessed for relationships within signaling networks.

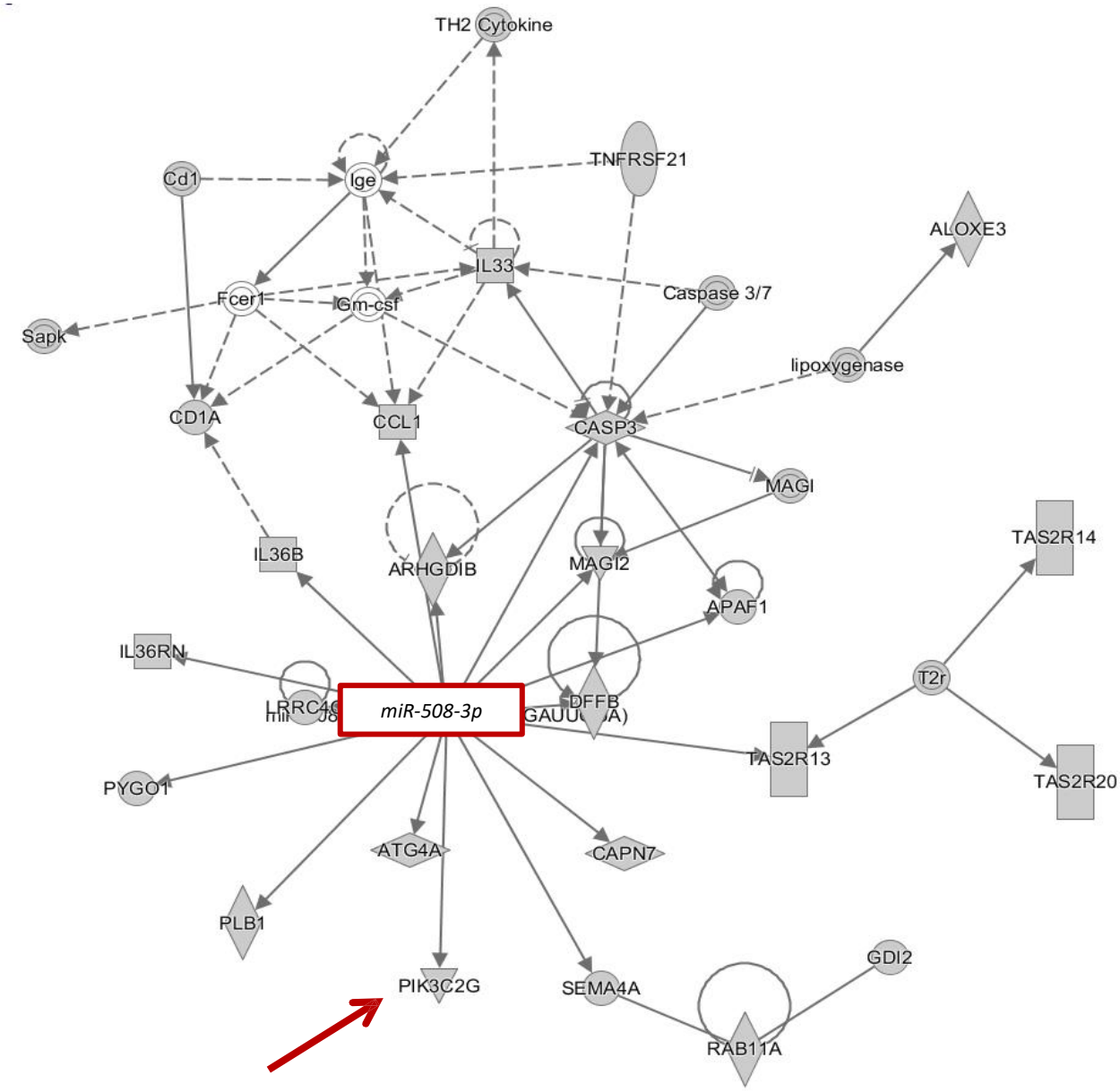

Figure 17. MiR-508-3p signaling pathway via IPA. MiR-508-3p directly targets PIK3C2G.

Identification of potential targets of *mir-508-3p* suggests that its mechanism could be analyzed via flow cytometry to examine its role in apoptosis and cell cycle transitions. A mechanism for *miR-1193* is less clear due to lack of pathway information; however, assessment of cell transformation via targeting of *LRRC1* could be analyzed by measuring its impact on colony formation in soft agar. Confirming targeting and mechanisms of these miRNAs could provide a suggestion of their clinical efficacy.

Future Directions

To summarize, *miR-508-3p* and *miR-1193* appear to be promising biomarkers in patients experiencing second biochemical recurrence, yet further study is warranted. Targets of each miRNA should be assessed via Western Blot and/or luciferase assay to confirm results of *in silico* searches. Mechanisms suggested by these targets should be assessed experimentally including flow cytometry to determine cell cycle transitions. Other mechanisms could be assessed through mitotic catastrophe and additional quantification of γ-H2AX foci in irradiated cells. Further, RNA sequencing of radiation resistant and radiation sensitive cell lines is in progress, but results are pending. Results should be analyzed for differences between radiation resistant and sensitive cell lines for further elucidation of mechanisms of radiation resistance.

Conclusion

The current study represents a functional screen of seven miRNAs previously correlated with second biochemical recurrence in PCa patients. Results of various *in vitro* assays indicated that *miR-508-3p* and *miR-1193* may act as tumor inhibitors in prostate cancer, potentially by regulating response to radiation. However, downstream mechanisms and confirmed targets of these miRNAs remain elusive, and further follow-up study is needed to fully characterize the role of *miR-508-3p* and *miR-1193* as biomarkers and potential therapeutic targets in PCa.

Acknowledgements

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